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GENE MAPPING STUDIES USING PIG-MOUSE SOMATIC CELL HYBRIDS

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DEDICATION

This thesis is dedicated to my wife, Karen, who stood by me and shared my moments of despair and triumph with remarkable decorum.

ABSTRACT

Pig lymphocytes were fused with mouse cells deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT-), by treatment with polyethylene glycol (PEG). Thirty permanent, vigorous clones of mononucleate, hybrid cells were studied. The pig and mouse chromosomes were distinguished with ease by conventional techniques. Hybrid clones lost pig chromosomes while retaining mouse chromosomes. The presence of nucleolus organizer regions (NORs) in pig chromosomes 8 and 10 is confirmed. These were identified in pig lymphocytes by their distinctive reaction with silver nitrate. The pig NORs of pig-mouse clones did not react with silver nitrate, but the reaction of the mouse NORs was undiminished. In the absence of any evidence of deletion from chromosome 10 this is interpreted to mean that the ribosomal genes of pig NORs are present, but are not transcribed, in these pig-mouse clones. Three enzymes glucose-6-phosphate dehydrogenase (G-6PD), HPRT, and alpha-galactosidase (GLA), are syntenic and are assigned to the X-chromosome of the pig. This agrees with assignments made for other mammals. One enzyme, dimeric superoxide dismutase (SOD-1), is assigned to chromosome 9. This is the first assignment of an enzyme to an autosome of a domestic or agricultural animal, and the fourth assignment of SOD

activity to an autosome. The fusion of pig lymphocytes with HPRT- mouse cells by exposure to PEG, and the culture of the hybrid cells in medium containing hypoxanthine, aminopterin, thymidine, and glycine, are efficient steps toward the mapping of pig genes. Mapping is facilitated by the vigorous growth of the hybrid cells, the distinctive character of the pig chromosomes, and the early preferential loss of the pig chromosomes.

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LIST OF ABBREVIATIONS

Ak-2	adenylate kinase-2
APRT-	adenine phosphoribosyltransferase deficient
A-T	adenine-thymine
ATPase	adenosine triphosphatase
AVG	anti-viral gene
AVP	anti-viral protein
cm	centimeter
DC	direct current
DEAE	diethylamino ethyl
df	degrees of freedom
DNA	deoxyribosenucleic acid
dTMP	deoxythymidylic acid
dUMP	deoxyuridylic acid
EDTA	ethylenediaminetetraacetate
Eno-1	enolase-1
g	relative centrifugal force
GALK	galactokinase
Gc	group-specific component
GLA	alpha-galactosidase
gm	gram
G-6PD	glucose-6-phosphate dehydrogenase
Gpd-1	hexose 6-phosphate dehydrogenase
HAT	selective culture medium
HBSS	Hank's Balanced Salt Solution

Het	heteropolymer
HJV	Sendai virus
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HPRT-	hypoxanthine-guanine phosphoribosyltransferase deficient
IDH	isocitrate dehydrogenase
IfRec	interferon receptor
IMP	inosine monophosphate
IP	intraperitoneal
M	molar
mA	milliampere
mg	milligram
ml	millilitre
mM	millimolar
MS	mean square
MU	map unit, the frequency of crossovers between two genetic loci in percent
NBT	Nitro-Blue tetrazolium
nM	nanomolar
NOR	nucleolus organizer regions
OTC	ornithine transcarbamylase
p	probability
PEG	polyethylene glycol
6-PGD	6-phosphogluconate dehydrogenase (human and pig)

Pgd	6-phosphogluconate dehydrogenase (mouse)
PGK	phosphoglycerate kinase
Pgm-2	phosphoglucomutase-2
PLR	pig lymphocyte-RAG hybrids
PMS	phenazine methosulfate, or 5-Methyl-phenazinium methyl sulfate
PP	pyrophosphate
PRPP	phosphoribosyl pyrophosphate
RAG	mouse cell line derived originally from a BALB/cd mouse
rRNA	ribosomal ribonucleic acid
S	Svedberg unit
SD	standard deviation
SDH	sorbitol dehydrogenase
SE	standard error
SOD	superoxide dismutase
SOD-	superoxide dismutase deficient
TK	thymidine kinase
TK-	thymidine kinase-deficient
TPA	12-O-tetradecanoylphorbol-13-acetate
TPN	triphosphopyridine nucleotide
TPNH	reduced TPN
ts	temperature-sensitive
ul	microlitre
V	volt

INTRODUCTION

I. Assignment of Genes to Chromosomes.

The fusion of somatic cells of different origins, and the fusion of their nuclei, to form permanent clones of mononucleate, hybrid cells was first reported in 1960 (Barski et al., 1960). Cells from different species hybridize (Ephrussi and Weiss, 1965), but there may be an early loss of some of the chromosomes of one or both species (Ephrussi and Weiss, 1967). In some combinations, the early loss is preferential for the chromosomes of one species, but the surviving chromosomes are relatively permanent and stable. The preferential loss of human chromosomes and gene products from human-mouse hybrids indicated that these hybrids could be used to map human genes (Weiss and Green, 1967). Such hybrid cells have been used to assign human genes to chromosomes (Greschik et al., 1972a, 1973; Ricciuti and Ruddle, 1973; Ruddle and Creagan, 1975; Minna et al., 1976), and to establish linkage between human genes (Boone and Ruddle, 1969; Ruddle, 1973; Tan et al., 1973).

The usefulness of these hybrids is attested by the speed with which they have expanded the map of human genes. With the aid of pedigrees, at least one structural-gene locus is now assigned to each human chromosome (McKusick and Ruddle, 1977); approximately 110 loci are assigned to autosomes and about 100 to the X chromosome. For many loci, the assignment is to a defined region of a particular chromosome (Ricciuti and Ruddle, 1973; Shows and Brown, 1975). Cell hybridization has been used to map other species (Pearson and Roderick, 1979). I have extended the method to the pig (Sus scrofa domesticus) by fusing its cells with mouse cells. This has allowed me to make the first assignment of an enzyme to an autosome of a domestic animal. This modest success and the expectation of more valuable information are due to the contrasts of pig and mouse chromosomes, the vigor of pig-mouse hybrids, and the preferential loss of pig chromosomes from these hybrids.

(A) Fusion

Fusion of somatic cells occurs in nature. The best-known is the fusion of myoblasts to form the synkaryons of striated muscle. Chicken myoblasts can fuse with mouse myoblasts in vitro to form heterokaryons (Wilde, C. E., 1958). Multinucleate cells are characteristic of some pathologies; some arise as a result of bacterial or viral infection (Enders and Peebles, 1954; Okada, 1958). Thus it was known before 1960 that cells which fuse with their own kind can fuse with similar cells from other species, and it was also known that multinucleate cells can arise from mononucleate cells under pathologic conditions. It was not clear, however, that different kinds of cells from different species could fuse. A demonstration of the fusion of cells of different origins appeared in 1965 (Ephrussi and Weiss, 1965; Harris and Watkins, 1965), shortly after the first evidence that spontaneous fusion occurs in mixed cultures (Littlefield, 1964). This stimulated the search for ways to increase the frequency of cell fusion.

Okada was the first to fuse cells experimentally (Okada, 1958, 1962). He used a myxovirus (HJV, also known as Sendai virus) which is still popular although other viruses are effective (Poste, 1972; Barski, 1970). Cells can also be caused to fuse using simple chemicals. Lysolecithin was used to fuse chicken erythrocytes (Poole et al., 1970), mouse fibroblasts (Croce et al., 1971; Ahkong et al., 1973), hamster cells (Gledhill et al., 1972), and rabbit sperm (Gledhill et al., 1972). Glycerol monooleate was used to fuse erythrocytes (Ahkong et al., 1973), and hamster fibroblasts (Cramp and Lucy, 1974). Liposomes containing various phospholipids were used to fuse mammalian cells (Papahadjopoulos et al., 1973). The frequency of fusion is comparable to that obtained with Sendai virus. Polyethylene glycol (PEG) was used to fuse plant protoplasts (Kao and Michavluk, 1974), hamster, mouse, and human cells (Pontecorvo, 1975), and cells of Drosophila melanogaster (Bernhard, 1976). PEG is an important agent because it is non-toxic in concentrations as high as 50 percent.

Human cells can be fused by microsurgery (Diacumakos and Tatum, 1972). Surgical fusion has important theoretical advantages. The needle, unlike chemicals or viruses, can be removed with certainty once fusion is achieved. The major disadvantages of the surgical technique are the time, practice, and labour it requires.

(B) Hybridization

The ease of fusion, the abundance of methods, and the diversity of cells show that experimental fusion does not depend on the biological specificity of the cell surface. Fusion may not, however, be permanent and productive; the binucleate cell may not survive long enough to be recognized. A fusion goes unrecognized if the binucleate reverts or does not grow well. The significance of fusion follows from the vigor and stability of mononucleate clones whose chromosomes disclose the fusion of dissimilar nuclei.

The isolation of these hybrid clones is the best proof of fusion, but is presumed to underestimate their frequency. The best measure of the error comes from the microsurgical fusions of human cells. Three of every four fusions, which could be isolated, failed to proliferate adequately (Diacumakos, 1973). Non-proliferation means loss which may or may not reflect a disappearance of essential genes by loss of whole chromosomes or parts of chromosomes. Whatever the case, chromosome and gene loss does occur early in the lives of clones, but less frequently in established clones (Ephrussi and Weiss, 1967). The perception of this phenomenon illuminated the experimental potential of hybridization for genetic studies and quickened the growth of the technology.

The isolation of a clone from a mixed culture depends on its ability to outgrow other cells and other hybrids. The yield of hybrid clones can be increased by selecting parental cells that cannot proliferate in media which allow the proliferation of the hybrid. Appropriate choices are parental cells that cannot survive in culture (e. g., normal lymphocytes), or have suffered a mutation which restricts an important metabolic process (e. g., nucleotide synthesis). The value of this approach was demonstrated by fusion of cells deficient in thymidine kinase (TK- cells) with cells deficient in hypoxanthine-guanine phosphoribosyltransferase

(HPRT- cells) (Littlefield, 1964). The TK- cells were selected by their resistance to 5′-bromodeoxyuridine and HPRT- cells by their resistance to 8-azaguanine. Resistant cells do not incorporate these unnatural bases into their DNA. Such cells do not proliferate in a medium (HAT) containing hypoxanthine, aminopterin, thymidine, and glycine. Conversely, the hybrid can proliferate because it inherits HPRT from the TK- parent and TK from the HPRT- parent and can circumvent the effects of aminopterin. This inhibitor has one incidental and two principal roles, both of which are due to a decline in tetrahydrofolate mediated by inhibition of dihydrofolate reductase. The incidental role is the inhibition of serine hydroxymethyltransferase which converts serine to glycine. This is countered by exogenous glycine. The principal roles are the inhibition of thymidylate synthetase, which converts deoxyuridylic acid (dUMP) to deoxythymidylic acid (dTMP), and of phosphoribosylaminoimidazole-carboxamide formyltransferase, which controls a path to inosine monophosphate (IMP), necessitating the use of the salvage pathways for the synthesis of thymidylate and purine nucleotides. A similar system uses TK- cells in combination with cells deficient in adenine phosphoribosyltransferase (APRT- cells) which are resistant to alanosine (Kusano et al., 1971; Tischfield and Ruddle, 1973). The basis of these complex interactions has been schematized (Fig. 1). TK- and HPRT- mouse cells fuse with cells from other species. HPRT- cells fused with human lymphocytes, in HAT medium, to yield hybrid clones (Miggiano et al., 1969; Ruddle et al., 1970; Tischfield and Ruddle, 1973). Lymphocytes do not normally attach or proliferate in culture and are removed when the old medium is replaced. In this thesis I describe the isolation of permanent hybrids of pig lymphocytes with HPRT- mouse cells (RAG cells).

Cells of the Chinese hamster have relatively few chromosomes and are well-suited to karyologic studies. Some mutagens, such as d-bromodeoxyuridine (plus visible light), produce mutant cells with special nutritional requirements (Puck and Kao, 1967). Some of these cell lines have specific requirements for adenine (Kao and Puck, 1972), glucose (Sun et al., 1974), glycine (Chu et al., 1972; Jones et al., 1972; Kao and Puck, 1972), inositol (Kao and Puck, 1972), proline (Kao and Puck, 1972), and uridine (Chu et al., 1972). In addition there are lines with requirements for purine or a combination of glycine, hypoxanthine, and thymidine (Chu et al., 1972). One line which requires glycine is deficient in serine hydroxymethylase (Jones et al., 1972), and the line which requires glucose cannot utilize galactose because of a deficiency of galactose-1-phosphate uridyltransferase (Chu et al., 1972). Hybrids can be recovered from media which lack the appropriate nutrient.

Mutant lines have been obtained from hamster cells selected in other ways. Temperature-sensitive (ts) mutants of BHK-21 cells were used to produce hybrids insensitive to the temperature of selection (Meiss and Basilico, 1972). One ts mutant affects DNA synthesis (Smith and Wigglesworth, 1973), and another affects purine transport (Harris and Whitmore, 1974). These ts mutants are recessive in the sense that they yield insensitive hybrids, which synthesize DNA and transport purine. Hamster and human cells are sensitive to ouabain (strophantin G), a glycoside which inhibits Na^+K^+-ATPase. Ouabain-resistant mutants were isolated from cultures of hamster cells and fused with sensitive cells to produce insensitive hybrids (Baker et al., 1974). Mouse cells resist concentrations 10^4 times those which kill human cells. Ouabain-resistant, HPRT-, mouse cells were fused with ouabain-sensitive, HPRT+, human fibroblasts to produce ouabain-resistant hybrids (Kucherlapati et al., 1975). It is clear that hybridization can be applied to many kinds of cells to answer many different questions.

(C) Chromosomes

A few chromosomes of humans and other mammals have been known by name, or number, for many years. The Y chromosome of some mammals was the easiest to identify. Using conventional staining techniques some autosomes could be discriminated by the location of the centromere, the number and positions of constrictions, the presence or absence of a terminal satellite, the shape, and the size. The human chromosomes we know as 1, 2, 3, 16, 17, 18, and Y were identified by those criteria. The identifications were based on the joint use of agents which destroy the mitotic spindle (colcemid), spread the metaphase plate (hypotonic KCl), and fix the chromosomes to a slide while removing most cellular matter (methanol-acetic acid). The ability to discriminate further was greatly improved by banding techniques (Caspersson et al., 1968, 1970; Wang and Federoff, 1972). The bands produced by two methods, Q-banding and G-banding, are termed Q-bands and G-bands meaning that there are transverse segments which fluoresce (Q-bands) or stain (G-bands) strongly or weakly (International System for Human Cytogenetic Nomenclature, 1978). The pattern of Q-bands is the same as the pattern of G-bands, for any one chromosome. The banding pattern is the same for the members of a homologous pair, is very similar in all tissues, and is more consistent than the intensities of the bands or the lengths of the chromosomes. In general, autosomes are numbered in descending order of length and grouped according to length and the position of the centromere. Standardization is facilitated by photography. The images of the chromosomes are cut from the print of a metaphase spread and arranged by length and morphology; the sex chromosomes are the last in a karyotype.

(D) Gene Assignment

For a particular species, the unambiguous identification of whole chromosomes, and of some segments of chromosomes, allows us to follow their fates after hybridization. Rat-mouse hybrids lose rat chromosomes (Weiss and Ephrussi, 1966), mouse-Chinese hamster hybrids lose mouse chromosomes (Scaletta et al., 1967), mouse-Syrian hamster hybrids lose mouse chromosomes (Migeon, 1968), human-mouse hybrids lose human chromosomes (Weiss and Green, 1967; Boone and Ruddle, 1969) human-Chinese hamster hybrids lose human chromosomes (Jones et al., 1972; Sun et al., 1974), mosquito-human hybrids lose mosquito chromosomes (Zepp et al., 1971), chicken-mouse hybrids lose chicken chromosomes (Schwartz et al., 1971), and chicken-Chinese hamster hybrids lose chicken chromosomes (Kao, 1973). The cell line whose chromosomes are lost preferentially after hybridization is referred to as "the recessive cell line". The loss of chromosomes may not be restricted to those of one origin; mouse and hamster chromosomes were lost from mouse-Chinese hamster hybrids (Scaletta et al., 1967; Handmaker, 1971; Labella et al., 1973) and mouse-Syrian hamster hybrids (Wilblin and MacPherson, 1973).

The concordant presence, or absence, of a chromosome and a phenotype is the basis of gene assignments. Assignment is usually made by comparing metaphase spreads (20 to 30) from different clones (5 to 10) with a test for the phenotype, e. g., an enzyme activity. Further, the deletion or loss of part of a chromosome, or the translocation of part to another chromosome, permits assignment of a gene to the deletion or translocation, or to the part which remains with its native centromere. Ionizing irradiation has been used to increase the frequency of deletions in human cells prior to their fusion with mouse cells (Goss and Harris, 1977); human cells are the recessive cells in these human-mouse hybrids. When used with HPRT- mouse cells this system selects for hybrid cells which retain that part of the human X-chromosome carrying the HPRT gene, and which may express human genes syntenic with HPRT.

Many genes are assigned to the X-chromosome of the mouse and some are assigned to each autosome (Womack, 1980; Davisson and Roderick, 1980). The genes are assigned by chromosome number, rather than linkage group, but the assignments represent a synthesis of karyotypic and linkage data. The data for most other mammals is fragmentary. Four linkage groups are known for the pig (Fig. 2): the locus for the K blood group is 4 map units (MU) from that for heme-binding globulin (Imlah, 1965), the C blood group is 6.0 MU from the J blood group (Andresen, 1966a), the I blood group is 2.5 MU from serum amylase (Andresen, 1966b), and the H blood group is between phosphohexose isomerase (2.6 MU) and 6-phosphogluconate dehydrogenase (6-PGD) (3.4 MU) (Andresen, 1971). None of these loci or linkage groups have been assigned to a pig chromosome.

II. Assignment of Genes to Pig Chromosomes

The objective of this thesis is the efficient assignment of genes to pig chromosomes. This objective has several stages: the discrimination of pig from mouse chromosomes, establishment of vigorous pig-mouse hybrids, characterization of chromosome loss, and detection of concordance of pig chromosomes with pig enzymes. I was able to discriminate all pig chromosomes from mouse chromosomes. Vigorous pig-mouse hybrids were obtained from HAT medium following PEG fusion of pig lymphocytes and RAG cells (HPRT- mouse cells). These hybrids lost pig chromosomes and retained mouse chromosomes. The presence of nucleolus organizer regions (NORs) in pig chromosomes 8 and 10 is confirmed. Three enzymes are assigned to the X-chromosome and one is assigned to chromosome 9. This is the first assignment of an enzyme to an autosome of a domestic, or agricultural, animal by somatic cell hybridization. The NOR of pig chromosome 10 is not visible in the hybrid cells which retained this chromosome. This may mean that the ribosomal genes of pig NORs are not transcribed in the hybrid, an interpretation for which there is ample precedent. I interpret my work to mean that it will be possible to assign many genes to pig chromosomes by fusion of pig lymphocytes with mouse cells, and the application of current techniques.

MATERIALS AND METHODS

(A) Cells

Mouse cells from a cell line (RAG) deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT-) (Klebe et al., 1970), were obtained from Linda Pasztor (University of Oregon). The RAG cell line is a nonreverting 8-azaguanine-resistant line derived from a spontaneous renal adenocarcinoma in a BALB/cd mouse of unspecified sex. Pig lymphocytes were obtained from an adult male pig of the Canadian Lacombe breed (Lin et al., 1976). Ten ml of heparinized pig blood were diluted with an equal volume of Hank's Balanced Salt Solution (HBSS) (Flow Labs., Mississauga, Ont.), pH 7.0, and layered above 20 ml of Ficoll-Hypaque solution. The latter was prepared from 9 gm of Ficoll (Pharmacia, Dorval, Quebec), 30 ml of sodium Hypaque (Winthrop Laboratories, Aurora, Ont.) and 120 ml of water. After centrifugation (280 X g, 40 min, room temperature), the cells were washed two times in HBSS (140 X g, 10 min). Except where stated otherwise all water used was double distilled in glass.

(B) Cultures

RAG cells were maintained in Ham's F-10 medium (Grand Island Biological, Burlington, Ont.) brought to 16 percent fetal calf serum (16 parts serum plus 84 parts medium) (Flow). The medium used for short-term cultures of pig lymphocytes was prepared from 10 ml Ham's F-10 medium, brought to 16 percent fetal calf serum, and 1 percent L-glutamine (Grand Island). To this I added 0.1 ml phytohaemagglutinin (Wellcome Reagents, Beckenham, U. K.) and 0.5 ml heparinized pig blood as a source of lymphocytes (Lin et al., 1976). These were cultured three days at 37°C and karyotyped. The selective medium, Ham's F-10 plus hypoxanthine, aminopterin, and thymidine (HAT), was prepared from Ham's F-10 medium brought to 16 percent fetal calf serum, 1×10^{-4} M hypoxanthine (Sigma Chem. Co., St. Louis, Mo.), 4×10^{-7} M aminopterin (Sigma), and 1.6×10^{-5} M thymidine (Sigma) (Littlefield, 1964).

(C) Hybridization

Ten ml of a suspension of RAG cells (10^5 cells/ml) and 10 ml of a suspension of pig lymphocytes (10^5 cells/ml), both in F-10 medium, were mixed and centrifuged (140 X g, 10 min). The pellet was suspended in 0.5 ml of a mixture of 5 gm PEG (Matheson, Coleman, and Bell, Cincinnati, Ohio) and 5 ml HBSS, pH 7.0 (1 min, 37°C). This was diluted to 10 ml with F-10 medium. Aliquots (0.5 ml) were added to 75 cm² culture flasks containing 10 ml each of HAT medium. Clones were isolated three to four weeks later in stainless steel cylinders (Ham and Puck, 1962). Primary clones were propagated in HAT medium until the numbers were adequate for sub-culturing. Secondary hybrid clones were maintained in regular F-10 medium. The permanent hybrid clones are identified as PLR 1, PLR 2, etc.

(D) Chromosome Preparation

Mouse chromosomes were prepared from the bone marrow of a BALB/cd female. The mouse was killed by cervical dislocation five hours after an IP injection of 50 ug (0.5 ml) colcemid (Grand Island). The ilia were removed, cut across the ends, and flushed with 2 percent sodium citrate. The marrow was centrifuged (140 X g, 10 min), dispersed in one to three ml 0.075 M KCl, and incubated (12 min, 37°C). This was centrifuged (140 X g, 8 min) and the cells were dispersed in fixative, three parts methanol plus one part acetic acid. One day later this was centrifuged (140 X g, 10 min) and the cells were dispersed in fresh fixative. Microscope slides were covered with cold water (4°C) and two drops of the cell suspension were dropped onto each slide. The slides were dried over a Bunsen burner.

Chromosomes were prepared from growing cultures of RAG, pig, and hybrid cells. Two hours after exposure to 0.1 ug/ml colcemid, the cells (10^7) were collected, exposed to 0.075 M KCl (15 min), fixed in methanol-acetic acid, and left overnight. The old fixative was replaced, two drops of cell suspension were dropped onto a chilled microscope slide, and the slide was air-dried as described above.

(E) Chromosome Banding

RAG, pig, mouse, and hybrid cell chromosomes were stained to show G-bands (Wang and Federoff, 1972). One ml of 5 percent trypsin (Difco Labs., Detroit, Michigan) in water was mixed with 20 ml of Puck's saline (136.0 mM NaCl, 4.0 mM KCl, 0.5 mM Na_2HPO_4, 11.0 mM sucrose) and 20 ml of EDTA-saline solution (5.0 mM disodium EDTA in 145.0 mM NaCl). The pH was adjusted to pH 8.0 with 0.1 M $NaHCO_3$. Slides were treated with trypsin (20 to 35 sec, room temperature) rinsed quickly in 70, 95, and 100 percent ethanol, and dried in air. Slides were stained (2 min, room temperature) in 1.0 ml Giemsa (Harleco, Gibbstown, N. J.) dissolved in 40 ml of Sorenson's buffer (0.07 M Na2HPO4-KH2PO4, pH 6.8).

Some slides were processed to show Q-bands (Lin et al., 1978). A slide was treated (20 min) with 0.128 mM actinomycin-D (Sigma), rinsed briefly in water, placed (10 min) in 0.05 ug/ml Hoechst 33258 (Behring Diagnostics, Montreal, Quebec), and air-dried. A few drops of sodium phosphate buffer (0.1 M, pH 4.5) were added to the slide. A coverslip was applied and sealed to the slide with paraffin. The slide was left in the dark overnight and examined in the fluorescence microscope the next day.

(F) Chromosome Retention

The chromosome complement of a hybrid clone varies from one cell to another although all cells of a clone are derived from a single fusion event. The variation can be visualized with the aid of a simple graph (Allderdice et al., 1973). Graphs for five clones were drawn by counting the number of different pig chromosomes present in one cell, the number present in a second cell which were not present in the first, the number present in the third which were not present in either the first or second, etc., summing these numbers for "n" cells, and plotting the sum as a function of "n" (see pages 30 to 32).

(G) Nucleolus Organizer Regions

Some slides were stained to show the NORs (Howell et al., 1975). Solution A was prepared by dissolving 0.5 gm of $AgNO_3$ (Fisher Scientific, Calgary, Alberta) in 1.0 ml of water. Solution B was prepared by adding 4.0 gm $AgNO_3$ to a mixture of 5.0 ml water and 7.5 ml NH_4OH (29 percent NH_3, Fisher). Solution C was prepared from 50 ml of 3 percent formaldehyde by adjusting the pH to 7.0 with solid sodium acetate (Sigma) and readjusting it to 4.5 with formic acid (88 percent, Fisher). Solutions A and B were filtered through 0.22 um MILLIPORE filters (Millipore, Bedford, Mass) immediately before use. Three drops of solution A were placed on a slide, a coverslip was added, the slide was incubated (15 min, 55°C), washed briefly in water, and dried in air. Four drops of solutions B and C were placed on the slide, a coverslip was added, and the slide was incubated at room temperature. The slide was rinsed in water and, as soon as a light brown color became visible, stained in Giemsa

(1.0 ml Giemsa in 40 ml Sorenson's buffer); the sequence was reversed for some photographs. Some slides were stained for Q-bands and NOR sites. For staining followed by silver impregnation the slides were processed to show the G-bands or Q-bands, photographed, the coverslip was removed, and the slides were treated to show the NORs and rephotographed.

(H) Tetradecanoylphorpbol Acetate

Ten mg of 12-o-tetradecanoylphorbol-13-acetate (TPA) (Peter Borchert, Chemical Carcinogenesis, Eden Prairie, Mn.) were dissolved in 10 ml acetone (Solution D) and 62 ul of this were added to 100 ml of Ham's F-10 culture medium (Solution E; 1000 nM TPA). Four sub-confluent cultures from clones retaining pig chromosome 10 were treated with 1.0 ml solution E (Expt. 1) and four were treated with 2.0 ml (Expt. 2) to give TPA concentrations of 100 and 200 nM. The cultures were incubated in 5 percent CO2 in air (24 hrs, 37°C). The cells were recovered, Q-banded, and stained for NORs. At least 20 metaphase spreads of each clone were examined. Solutions lacking TPA were used in parallel as controls.

(I) Enzyme Separation

The medium of a RAG or hybrid culture was replaced with two ml of EDTA-Saline (136 mM NaCl, 5 mM KCl, 5 mM glucose, 4 mM $NaHCO_3$, 0.5 mM EDTA). After five minutes at room temperature the flasks were tapped gently on the side to detach the cells. Cells (10^7) were washed three times by centrifugation (140 X g, 10 min) in cold isotonic saline, then lysed in 0.2 ml 30 mM phosphate buffer, pH 7.0, by three cycles of freezing, in liquid nitrogen, and thawing, at room temperature. The clear supernatant (20,000 X g, 30 min, 4°C) was used for electrophoresis in starch gel. Extracts of pig lymphocytes and liver cells were prepared in the same manner.

Tests for four enzymes proved useful. The enzymes are glucose-6-phosphate dehydrogenase (G-6PD) (Goldstein and Lin, 1971), hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Nichols and Ruddle, 1974), alpha-galactosidase (GLA) (Harris and Hopkinson, 1976), and superoxide dismutase (SOD) (Nichols and Ruddle, 1973). A different pair of buffers, a bridge buffer and a gel buffer, was used for each enzyme. Sixty five grams of hydrolyzed starch (Sigma) were added to 500 ml of gel buffer and heated. This was poured into the gel mold and left at room temperature for at least four hours. Aliquots of the extracts (20 ul) were placed in wells 5 cm from the cathodal end of the gel, the wells were sealed with melted petroleum jelly (Fisher) and the gel was wrapped in Saran wrap, leaving the ends exposed.

The bridge buffer for G-6PD was 0.05 M phosphate-citrate, pH 7.4, and the gel buffer was a 10-fold dilution. The bridge buffer for HPRT was 0.027 M citric acid and 0.167 M K2HPO4, pH 6.8, and the gel buffer was was about a 25-fold dilution (1.21 mM and 6.07 mM). The bridge buffer for GLA was 0.44 M H3BO4 and 0.04 M LiOH, pH 7.0, and the gel buffer was 12.4 mM Tris, 3.3 mM citric acid, 3.6 mM H3BO4, and 0.33 mM LiOH, pH 7.0. The bridge buffer for SOD was 0.02 M EDTA, 0.5 M H3BO4, and 0.9 M Tris, pH 8.6, and the gel buffer was a 20-fold dilution. For SOD, a five-fold dilution of the bridge buffer was used in the anode chamber and a seven-fold dilution in the cathode chamber.

The G-6PD activities of the mouse and pig were separated by electrophoresis for 16 hours at 160 V and 16 mA, HPRT activities by 17 hours at 150 V and 15 mA, GLA activities by 16.5 hours at 150 V and 15 mA, and SOD activities by 19.5 hours at 210 V and 12.5 mA. All electrophoretic runs were done at 4°C in a vertical starch gel apparatus (Buchler Instruments, Fort Lee, N. J.) using a DC power supply (Buchler).

(J) Enzyme Detection

For G-6PD, each half-gel was incubated with substrate (37°C, 30 min; 5 mg glucose-6-phosphate, 25 mg $MgCl_2.6H_2O$, 3 mg TPN, 3 mg Nitro-Blue tetrazolium or NBT, 1 mg phenazine methosulfate or PMS, all Sigma) in 25 ml 0.05 M Tris-HCl buffer, pH 8.0, in the dark.

For HPRT, a sheet of DEAE ion exchange paper (Fisher) was placed on each half-gel, and incubated with substrate (37°C, 2 hrs; 50 ul hypoxanthine-8-^{14}C, specific activity, 42.4 mC/mM, New England Nuclear, Boston, Mass; 1.5 mg 5-phosphoribosyl-1-pyrophosphate or PRPP, Sigma; 30.0 mg of $MgCl_2$, Fisher) in 15.0 ml of 0.1 M Tris-HCl buffer, pH 7.4. The paper was soaked overnight in a large volume of 0.1 M $LaCl_3$ (Fisher) and 0.1 M Tris-HCl, pH 7.0, washed two hours in running deionized water, dried, and placed on X-ray film (X-Omat R film, Eastman, Rochester, N. Y) for one week or longer. The film was developed, placed on printing paper (Eastman), and exposed on a light box (1 sec).

For GLA, each half-gel was incubated with substrate (37°C, 2 hrs; 10 mg 4-methylumberilliferyl-alpha-galactoside, Sigma) in 20 ml 0.2 M phosphate-citrate buffer, pH 4.0. Each half-gel was flooded with 30 ml of 29 percent aqueous NH_3, illuminated with long-wavelength ultraviolet light, and photographed.

For SOD, each half-gel was incubated with substrate (15 min, room temperature; 50 mg MgCl2.6H2O, 6 mg NBT, 2 mg PMS) in 25 ml of 0.05 M Tris-HCl, pH 8.0, placed under a flood light for 30 minutes, and photographed immediately.

(K) Other Enzymes

Cell lysates were tested for six other enzymes: 6-PGD (Nichols and Ruddle, 1973), isocitrate dehydrogenase (IDH) (Nichols and Ruddle, 1973), ornithine transcarbamylase (OTC) (Baron and Buttery, 1972), sorbitol dehydrogenase (SDH) (Lin et al., 1969), phosphoglycerate kinase (PGK) (Beutler, 1969), and PRPP synthetase (Lebo and Martin, 1978). One enzyme, pig 6-PGD, could not be detected in any of 26 clones. The activities of IDH, OTC, and SDH were too weak to permit precise interpretation of the gels. PGK and PRPP synthetase were detected, but the pig and mouse enzymes migrated together at pH 7.5, for PGK, and pH 6.8, for PRPP synthetase. The effects of altering the pH were not tested.

RESULTS AND DISCUSSION

(A) The Pig Chromosomes of Hybrid Cells

Distinct chromosome bands were demonstrated in pig (Fig. 3 and 4), mouse (Fig. 5), RAG (Fig. 6 and 7), and hybrid cells (Fig. 8 and 9). The 20 different chromosomes of the male pig were discriminated and identified in pig lymphocytes (Fig. 3 and 4) and hybrid cells (Fig. 8 and 9; Table 1a). RAG cells contain two kinds of mouse chromosomes: intact chromosomes identical with those of the normal mouse cell, and marker chromosomes produced by translocation between mouse chromosomes. The mouse X-chromosome, all mouse autosomes, and some of the mouse marker chromosomes were discriminated and identified in RAG and hybrid cells (Fig. 6 to 9). The chromosomes of hybrids are easily identified as pig or mouse by the coloration of the centromeres, weak for those of the pig and intense for those of the mouse (Fig. 8 and 9). The intense fluorescence of mouse centromeres (Fig. 7 and 9), after treatment with actinomycin-D and Hoechst 33258, is attributed to the high density of adenine-thymine (A-T) base pairs (Hilwig and Gropp, 1972; Raposa and Natarajan, 1974; Jalal et ., 1976; Jorgensen et al., 1978). Karyotypes (Fig. 3 to 9) were arranged (Lin et al., 1980; the Reading Conference, 1980), and individual chromosomes of mouse origin were identified, according to current standards (Committee on Standardized Genetic Nomenclature for Mice, 1972; Hashmi et al., 1974). The cells of hybrid clones resembled RAG cells (Fig. 10), despite the retention of pig chromosomes. I use the terms "loss", or "negative", and "retention", or "positive", when referring to, or categorizing, clones. The terms "absence", "frequency", and "presence" are used to describe the metaphases of various cells, cell lines, or clones. "Absence" means that a particular type of chromosome occurs zero times in a particular metaphase, "presence" means that it occurs one or more times, and "frequency" means the proportion of metaphases in which the chromosome occurs one or more times. "Occurrence" is used to indicate the number of times a particular type of chromosome is found in one metaphase. Chromosome types are identified by numbers, from 1 to 19, for mouse autosomes, and from 1 to 18, for pig autosomes, or by the letters X and Y for the sex chromosomes. The number of "different" chromosomes means the number of different types, not the total number which includes replicates, e.g., the number of different types in the male pig is 20, but the total number of chromosomes is 38.

Examination of 14 to 22 metaphase spreads from each of 26 hybrid clones showed that 302 (58 %) of 520 (20 x 26) pig chromosomes were retained and 218 were lost (Table 1a). For the 26 hybrid clones, the mean number of different pig chromosomes was 11.62 (SD = 3.16; SE = 0.62; range = 5 to 18). For the 20 types of pig chromosomes, the mean number of positive clones was 15.10 (SD = 6.27; SE = 1.40; range = 3 to 26). The mean square (MS) is 9.90 for the number of different pig chromosomes per clone, and 37.29 for the number of clones per type (F = 2.98 for homogeneity of variance; $p < 0.05$). The number of different pig chromosomes per clone is less variable than the number of clones per type, which shows that retention and loss are not completely random. The more acrocentric types, 13 to 18, were retained more frequently than the non-acrocentric types, 1 to 12. Of 156 (6 x 26) acrocentrics, 121 (78 %) were retained and 35 were lost (Table 1a). Of 312 non-acrocentrics, 155 (50 %), were retained and 157 were lost. The number of acrocentrics retained does not differ significantly from the number of non-acrocentrics, but the 78 percent retention of acrocentrics is significantly greater (F= 22.43; df = 1 and 50; MSs are equal; $p < 0.001$). All 26 clones retained chromosome 16, most retained 13, and most lost 12, 3, and Y. Thirteen of the 26 clones combined these retentions and losses, keeping 16 and 13 and losing 12, 3, and Y. This is in good agreement with the expectation of 0.51, estimated from the mean retention of each type. This suggests that the retention of a particular acrocentric is not related to the loss of a particular non-acrocentric. A test for correlation shows that the number of different acrocentrics retained is independent of the number of different non-acrocentrics (p > 0.25). A set of seven clones was used to assign genes to the X-chromosome (Table 1b; PLR 1 is not the same as PLR 1 of Table 1a, PLR 6 is not included in Table 1a.). PLRs 1 to 7 of Table 1b and the first 11 of Table 1a, used to assign SOD to chromosome 9 (see pages 38 to 42), were examined separately to see if the ratio of acrocentrics to non-acrocentrics is the same in the different sets of clones used to make assignments. The ratios are 1.71 (86 % and 50 %) for the X-chromosome set, 1.59 (83 % and 52 %) for the chromosome 9 set, and 1.45 (67 % and 46 %) for the remainder of Table 1a. These comparisons show that most of the variability associated with chromosome type can be attributed to the difference between acrocentrics and non-acrocentrics, as groups or classes. Most of the variation among sets used for different purposes is due to the acrocentrics, to which I have made no assignments. Relatively little variation can be attributed to the non-acrocentrics as a group, to chromosome type, or to clone.

For RAG cells, the total number of intact and translocation, or marker, chromosomes was estimated from 20 G-banded metaphases (Fig. 11). The mean total of all chromosomes is about 60 (SE is about 0.6) and the mean total of marker chromosomes is about 13 (SE is about 0.8). The mean total of intact mouse chromosomes is 47, which includes replicates. The maximum for different types of intact mouse chromosomes is 21. Comparison of G-banded RAG and hybrid metaphases did not detect translocations between RAG and pig chromosomes, or between one pig chromosome and another.

The variation among RAG cells is complex. There may be one, two, or several chromosomes of any one type, including markers (Fig. 6 and 7). Comparison of these two figures shows that only six of the 19 types of intact chromosomes occur the same number of times; chromosomes 12 and 16, once, 6 and 11, twice, 17, three times, and 9, four times. Variable representation of these and other chromosomes was seen in other RAG metaphases. Such variation is characteristic of RAG cells and permanent cell lines in general. The hybrid clones show cell-to-cell variation for all chromosomes (Fig. 8 and 9). RAG chromosomes 16, once, and 6, twice, are the only intact chromosomes of mouse origin which occur the same number of times in the two RAG karyotypes (Fig. 6 and 7) and the two hybrid karyotypes (Fig. 8 and 9). The cell-to-cell variation for pig chromosomes was estimated for clones PLR 9 (Fig. 12) and PLR 8 (Fig. 13). No one type of pig chromosome was present in all the metaphases from either clone. The highest frequencies, 80 to 90 percent, were those for pig chromosomes 13, 15, and 16 and the lowest were those for 1 to 7, and 12. Since no chromosome is present in all metaphases of a clone, and some are present in only a few metaphases, it is evident that the "loss" or "retention" of a pig chromosome must be based on the examination of an adequate number of metaphases. If, for instance, a chromosome is present in 20 percent of the metaphases it is necessary to examine 15 metaphases to conclude that it is completely absent, with less than a 5 percent chance of being wrong. If the level of probability selected is 1 percent, the requisite number of metaphases is 22. In general, it is not necessary to prove the complete absence of a chromosome. To establish "loss" it suffices to show that the chromosome is absent from more than 80 percent of the metaphases. The "retention" of a chromosome is based on the converse, recognition in 20 percent or more of the metaphases. Strictly applied, this arbitrary division means that the presence of a chromosome in three, or less, of 20 metaphase spreads is "loss" and its presence in four, or more, is "retention". Fortunately, cell-to-cell variation can be dealt with in a more satisfactory manner.

The variation among the cells of a clone was assessed in five clones, PLRs 13, 22, 31, 50, and 96 (Fig. 14), by construction of heterogeneity curves (Allderdice et al., 1973); the five clones were used in the assignment of SOD-1 to chromosome 9; PLR 31 is PLR 1 in Table 1a and is negative for SOD-1 and chromosome 9, and the other four are positive for both (see pages 38 to 42). Twenty metaphase spreads of each clone, excepting PLR 13 (19 metaphases), were examined. In the original report (Allderdice et al., 1973) the curves represent the sum of different chromosomes, e. g., the first metaphase spread may have five different pig chromosomes and the second may have two different pig chromosomes not present in the first, so that the first two points on the line would be 5 and 7. In this example it does not matter if the second metaphase spread has none, one, two, three, four, or all five of the different chromosomes seen in the first. If a curve is drawn for a clone on the basis of one series of 20 metaphase spreads it is likely to be irregular or jagged. If the curve is redrawn, after changing the order in which the metaphase spreads are represented, the new curve will differ from the first, but it is likely to be as irregular or jagged. A set of 20 of these irregular curves can be averaged to construct a smooth, mean curve with which to estimate the terminal slope. The terminal slope predicts the number of additional metaphase spreads which must be read to increase the number of different chromosomes by one. This manner of representation was introduced as a measure of the heterogeneity, or homogeneity, of the human chromosome complement of a human-mouse clone. When the terminal slope is close to 0.2 it indicates that five metaphase spreads must be read to increase the number of different chromosomes by one. The terminal slope can also be estimated by summing the number of different chromosomes in a different way, e. g., if the sequence is 5, 2 (not present in the first metaphase spread), and 1 (not present in the first or second metaphase spreads), the value for the first metaphase spread is 5, that for the second is 5 + 7 divided by 2, the third is 5 + 7 + 8 divided by 3, etc. Applied to the data of the original report (Allderdice et al., 1973) this abbreviated method gives a terminal slope close to 0.2, the slope obtained in the original report by 20 repetitions of the original method. With the abbreviated method the slopes for the pig-mouse hybrids are less than 0.2.

Reexamination of the original report discloses variation within a clone analogous to that we see between clones (Table 1a). The number of cells per human chromosome type was much more variable than the number of different human chromosomes per cell ($p < 0.005$). The greatest part of this variability was due to the almost complete absence of human chromosomes 1 to 9 and the relative abundance of the others ($p < 0.001$). In effect, the data for a heterogeneity curve may mimic the data for a population of separate clones; compare Table 1a with Table 1 in the original report (Allderdice et al., 1973). This suggests that any one clone may be made up of somewhat different sub-clones. We need to know how many metaphases must be read in order to compensate for this and we need an incisive means of comparing the intra-clonal variation of two clones. The mean heterogeneity curves appear to do this (Fig. 14). The curves tell us that 11 metaphase spreads give a good estimate of the number of different chromosomes, 15 improve this slightly, and 20 are only slightly more reliable than 15. Equally important, clones can be categorized and discriminated from each other by 11 metaphase spreads, e. g., PLR 22 versus PLR 31 (Fig. 14). The similarity of the terminal slopes for these two clones shows that the addition of more metaphases would not invalidate the quantitative comparisons based on 11 metaphase spreads. These observations justify the search for sets of clones composed of two subsets, one negative for a particular type of pig chromosome and one positive.

(B) The Nucleolus Organizer Regions

A segment of pig chromosome 10 (Fig. 15), and another in chromosome 8, was identified as an NOR, a site of ribosomal ribonucleic acid (rRNA) synthesis (Table 2). The identifications were made in lymphocytes from five pigs. The identification of an NOR may depend on its activity, i. e., the intensity of silver impregnation may reflect the intensity of rRNA synthesis. Failure to detect an NOR where expected may mean that it is present, but inactive, e. g., pig chromosome 10 is NOR- in hybrid cells. An NOR was detected in both No. 10 chromosomes, but in neither No. 8, of 24 metaphase spreads from pig No. 4 (Fig. 15). Detection was less consistent for the lymphocytes of other pigs, particularly pig No. 3. Consequently, male pig No. 4 was selected as the source of lymphocytes for hybridization.

Four clones, PLRs 8, 11, 20, and 52, were used in a search for pig NORs (Table 3). For 87 metaphase spreads the total number of RAG (mouse) NORs was 865; the total for pig NORs was zero. The number of pig No. 10 chromosomes identified was 111. Thus, an NOR was detected in none of 111 No. 10 chromosomes. NOR- No. 10 chromosomes were identified in spreads which contained RAG NORs (Fig. 16 and 17). An attempt to visualize the NOR of pig chromosome 10 by reactivation with TPA (Soprano and Baserga, 1980) did not succeed (Fig. 18 and 19; Table 4). The abundance of RAG NORs shows that there is no general suppression of NOR activity which could account for the non-detection of pig NORs. Since there is no indication of deletion from No. 10 I infer that the NOR of pig chromosome No. 10 is present, but inactive. Previous reports show that the non-detection of NORs in hybrid cells is associated with the preferential loss of chromosomes from one parental species. The species whose chromosomes are lost, preferentially, is the species whose NORs are inactivated (Soprano et al., 1979). The pig is the losing or "recessive" species for these pig-mouse hybrids and it is the pig NORs which cannot be detected. Presumably, the explanation for the non-detection of NORs in other hybrid cell lines, when learned, will also explain the non-detection of pig NORs in pig-mouse clones. The best evidence for the presence of the NOR in NOR- hybrids is reactivation by two very different agents SV-40 virus (Soprano et al., 1979) and TPA (Soprano and Baserga, 1980). My attempt to reactivate pig NORs with TPA did not include a positive control, i. e., a hybrid clone whose negative NORs had been reactivated in previous experiments of others. It is possible that my use of TPA was faulty. I do not infer that the NOR site of pig chromosome No. 10 is lost from PLRs 8, 11, 20, and 52, and the other PLRs, although I have not proved the opposite, that it is retained.

(C) G-6PD, HPRT, GLA and the X-Chromosome

The histochemical basis for the detection of G-6PD activity in starch gel is as follows: the staining mixture consists of the substrate, glucose-6-phosphate, the necessary cofactor, TPN, an electron transport carrier, PMS, and a colourless dye, NBT. In the presence of G-6PD, TPN is reduced to TPNH. As TPNH is formed, it reduces the PMS, which in turn reduces the NBT. The latter is converted to a blue precipitate, formazan. The formazan reveals the location of G-6PD.

The detection of HPRT activity in starch gel depends on depyrophosphorylation and ribosylation. The enzyme converts PRPP and radiolabelled hypoxanthine into labelled IMP and pyrophosphate (PP). Labelled IMP is precipitated by lanthanum chloride and having acquired a negative charge binds to the ion-exchange paper. Excess hypoxanthine is removed with Tris-HCl buffer. Exposure of the ion-exchange paper to X-ray film locates the labelled IMP and the enzyme. Direct printing of the X-ray films gives convenient replicas.

Another unnatural substrate is used to locate GLA. GLA cleaves D-galactose from 4-methylumbelliferyl-alpha-galactoside, yielding 4-methylumbelliferone, which fluoresces brightly between pH 10 and pH 12.

Of seven hybrid clones tested for pig G-6PD, HPRT, and GLA, five (PLRs 1, 2, 3, 6, and 7) exhibited all three activities (Table 1b), and the pig X-chromosome was present in 43 to 86 percent of the metaphase spreads. PLRs 4 and 5 did not exhibit any of the three activities, and the X-chromosome was present in just 4 percent of the PLR 4 metaphases and in none of the PLR 5 metaphases. The retention and loss of enzyme activity was judged by comparison with appropriate controls (Fig. 20 to 23). The retention and loss of the pig X-chromosome was judged by the closeness of the frequencies, 43, 4, and zero percent, to the reference percentile (20 %) for "retention" versus "loss". The possible qualification that an X-chromosome may be retained and inactive, if derived from a female pig, was anticipated by using a male pig (No. 4) as the donor of lymphocytes for hybridization. The concordance of the enzymes with the X-chromosome is significant by Fisher's exact test (p = 0.048).

Pig G-6PD and GLA formed electrophoretic intermediates with the RAG enzymes (Fig. 20, 22, and 23). As expected, HPRT did not form an active intermediate (Fig. 21) because RAG cells are HPRT-. I have not included the HPRT tests for PLRs 4 and 5 in the figures. In fact, it is not clear how PLRs 4 and 5 could survive and grow in selective medium. Presumably, PLRs 4 and 5 lost the pig X-chromosome after transfer to maintenance medium, in which the loss would not be lethal. The alternative possibility is that RAG HPRT activity recovers to a marginally adequate level which I do not detect. I do not know of proof for the spontaneous recovery of RAG HPRT activity after hybridization, and I do not know if pig and mouse HPRT would form active intermediates. I have not included a positive test for mouse HPRT in the figures. I did not return PLRs 1 to 7 to HAT medium to prove retention and loss of pig HPRT. The formation of active intermediates of G-6PD and GLA shows that these pig and mouse enzymes can combine to form active heteropolymers. Enzyme intermediates, which must be heteropolymers, have been reported for other interspecific hybrids of somatic cells (Boone and Ruddle, 1969; Westerveld et al., 1972; Chapman and Shows, 1976; Garver et al., 1978; Heuertz and Hors-Cayla, 1978).

The concordant retention of G-6PD, HPRT, and GLA in PLRs 1, 2, 3, 6, and 7, and the presence of the pig X-chromosome in 43 percent or more of the metaphases, suggests that the three enzymes are syntenic and that their structural, or regulatory, genes are located in the X-chromosome. The acceptance of this depends on the converse, the loss of the three enzyme activities from PLRs 4 and 5 and the absence of the X-chromosome from more than 80 percent of their metaphase spreads. The activities of the three pig enzymes, in PLRs 1, 2, 3, 6, and 7, are much weaker than the activities obtained from pig tissues. This may be related to the frequency of the X-chromosome, which was present in 64 (43 to 86) percent of the metaphase spreads from PLRs 1, 2, 3, 6, and 7. This deficiency might account for the relatively weak activities of the pig enzymes. I did not return PLRs 1, 2, 3, 6, and 7 to HAT medium which should have increased the enzyme activities by eliminating sub-clones which had lost the pig X-chromosome. The effect of variation in the number and kind of mouse chromosomes was not examined. The data seem adequate proof for the assignment of G-6PD, HPRT, and GLA to the X-chromosome of the pig. In this respect the pig does not differ from other mammalian species (Ohno, 1969, 1973).

(D) Superoxide Dismutase and Chromosome 9

The term "superoxide dismutase" (SOD) identifies a group of enzymes which dismutate the superoxide anion, the oxygen molecule bearing a single negative charge. "Dismutation" means that two anions undergo mutual oxidation-reduction to form free oxygen and the peroxide anion. There are two principal forms of the enzyme, a cytosol dimer, which contains copper and zinc, and a mitochondrial tetramer, which contains manganese. The dimer resists organic solvents and is sensitive to cyanide, and the tetramer is sensitive to organic solvents and resists cyanide. The dimer usually migrates more rapidly during electrophoresis. Both enzymes have been identified in extracts of pig heart (Weisiger and Fridovich, 1973), and both should be present in pig cells which have mitochondria, i. e., the erythrocytes should have the dimer, but lack the tetramer because they lack mitochondria (Beckman et al., 1973). Lysates of pig lymphocytes contain two electrophoretically distinct SODs (Fig. 24). Neither cyanide nor organic solvents were used to inhibit SOD activity so that it is not immediately clear whether the two electrophoretic bands (Fig. 24) represent (anodal) dimeric SOD and (cathodal) tetrameric SOD, or two electrophoretic variants of the dimeric form. If the lymphocyte preparation was heavily contaminated it is possible that the cathodal band represents the tetrameric form; a common contaminant, the polymorphonuclear granulocyte, contains many mitochondria and is the richest source of tetrameric SOD. The most convenient source of the dimeric enzyme is the erythrocyte, another common contaminant. The identity of the pig SOD present in extracts of pig leucocytes (Fig. 24) is unproven. The occurrence, however, of an intermediate enzyme in PLRs 9 and 96 (Fig. 24), and the other positive clones, indicates that it is the dimeric form of pig SOD which is retained. This conclusion follows from, 1) the occurrence of three bands (Fig. 24), 2) the nearly equal spacing of the three bands, 3) the absence of the intermediate band from pig and RAG cells, 4) the alignment of the most cathodal band of clones with that of pig leucocytes, and 5) the intensity of the cathodal band of pig leucocytes. The only obvious interpretation is that the cathodal band of pig leucocytes represents pig SOD which also combines with RAG SOD to form the intermediate. The RAG SOD is the anodal or dimeric SOD of the mouse (Nichols and Ruddle, 1973) homologous with the dimeric SOD-1 of humans (Brewer, 1967); the tetrameric SOD of the mouse migrates toward the cathode (Nichols and Ruddle, 1973) and was not seen in my gels. The interpretation of the three bands from positive PLRs is very similar to the interpretation of bands from hybrids of Chinese hamster and mouse cells (Francke and Taggart, 1979), and from hybrids of human and Chinese hamster cells (Moore et al., 1977). Comparison with other studies (Weisiger and Fridovich, 1973) indicates that the four bands seen in preparations from pig leucocytes may correspond to those seen in preparations from other tissues of other species: an unnamed, vague band level with (+), band A just cathodal to RAG SOD, band B just anodal to (-), and band C just above the origin (Fig. 24). The named bands are variants of dimeric SOD. It seems most unlikely that the SOD retained by seven positive PLRs is the tetramer, which appears in many preparations from other species as a single, cathodal band D (Weisiger and Fridovich, 1973).

The retention and loss of the pig SOD, which I identify as band C of SOD-1 (Weisiger and Fridovich, 1973), is completely concordant with the retention and loss of pig chromosome 9 (Table 5); seven clones are positive for both and four are negative for both. The difference is statistically significant (Table 6). The Chi-square test ($p < 0.01$ after correction for the small numbers) is confirmed by Fisher's exact test ($p = 0.003$). Three questions are answered for the SODs of pig-mouse clones: 1) the chromosome identification, by two methods, 2) the identities of the SODs, and 3) the concordance of chromosome 9 and pig SOD-1. Two questions are unanswered: 1) the randomness of chromosome loss from the clones which lost pig SOD-1, and 2) the meaning of the concordance of chromosome 9 and pig SOD-1. I have no way of assessing randomness as such, but I have assessed the similarity of the 11 relevant clones (Table 5) to other clones. The 26 clones listed in Table 1a preferentially lost pig chromosomes 1 to 12, and preferentially retained pig chromosomes 13 to 18. The 11 clones of Table 5 are the first 11 clones of Table 1a (PLR 31 of Table 5 is PLR 1 of Table 1a; PLRs 1 and 6 of Table 1b and PLRs 8 and 11 of Tables 3 and 4 are not included in Table 1a; the total number of PLRs is 30, not 26.). The 11 clones tested for SOD-1 were compared with the 15 other clones of Table 1a. The 11 clones retained 83 percent of acrocentric chromosomes 13 to 18 and 52 percent of non-acrocentric chromosomes 1 to 12. The other 15 clones retained 74 percent of chromosomes 13 to 18 and 47 percent of chromosomes 1 to 12. The ratios of acrocentrics to non-acrocentrics are 1.59 and 1.58 (see page 28). The difference in the way the two sets of clones handle the two groups of pig chromosomes is non-significant. There is no reason to believe that clones positive for SOD-1 (9, 13, 16, 20, 22, 50, and 96) and those negative for SOD-1 (26, 31, 71, and 76) are unusual; PLRs 13 and 31 have very similar heterogeneity curves and 22, 50, and 96 differ by having more chromosomes (Fig. 14). The last question, the meaning of concordance, cannot be answered. The non-detection of pig SOD-1 in four clones lacking chromosome 9 may mean that chromosome 9 carries the structural gene or it may mean that it carries a regulatory gene.

CONCLUSION

The preferential loss of non-acrocentric pig chromosomes, and the preferential retention of acrocentric pig chromosomes, from pig-mouse hybrids is not without precedent. Nonrandom loss and retention have been suggested (Yoshida and Ephrussi, 1967; Santachiara et al., 1970).

Mouse-hamster hybrids may lose mouse chromosomes in groups (Marin, 1969; Marin and Pugliatti-Crippa, 1972), and human-mouse hybrids show preferential retention of some human chromosomes (Minna and Coon, 1974; Norum and Migeon, 1974). There may also be a preferential loss and retention of individual chromosomes; pig chromosome 12 was lost from 25 of 28 clones, and chromosome 16 was retained by all. Considering the small number of clones examined this difference must be regarded as random, but there are precedents. Human chromosomes 7 and 17 were retained by human-mouse hybrids (Croce et al., 1973), and certain human autosomes and the X-chromosome were retained preferentially by hybrids of human with Chinese hamster cells (Douglas et al., 1973). Mouse chromosome 11 was lost from all hybrids of the mouse with the Chinese hamster and chromosome 15 was retained by all (Francke et al., 1977; Kozak and Ruddle, 1977). Chromosome loss does not appear to be random with time. Most of the loss may occur in the first few days (Ephrussi and Weiss, 1967; Terzi, 1974) and the subsequent losses may not occur at a constant rate.

Several mechanisms have been proposed to explain the species specificity of chromosome loss. Initially it was thought that lost chromosomes are those which replicated too slowly. In other words, the generation times of the parental cells may influence the acquisition and retention of their chromosomes by hybrids (Kao and Puck, 1970). There are instances in which the chromosomes of the slower-growing parental cells are underrepresented (Weiss and Green, 1967; Scaletta et al., 1967; Kao and Puck, 1970; Koyama et al., 1970). Since some blood lymphocytes divide very slowly in the absence of specific stimulation, and I used blood lymphocytes as donors of pig chromosomes, I might have anticipated the preferential loss of pig chromosomes. However, the chromosomes of the slower-growing parental cell may not be rejected preferentially and the signals for replication seem to apply equally to all chromosomes in the hybrid cell (Labella et al., 1973). Other hypotheses attribute preferential loss and retention to the mitotic apparatus. The argument favouring some species-specificity for the mitotic apparatus is by analogy with the preferential loss of chromosomes from hybrids produced by interspecific fertilization (Terzi, 1974).

A NOR is found in pig chromosome 8 and another in pig chromosome 10. The incidence varies from pig to pig, as judged from the silver impregnation of metaphase spreads of lymphocytes stimulated in vitro with phytohemagglutinin. A particular male pig was selected as the donor of all lymphocytes used in hybridization because it gave consistent results; the NOR of chromosome 8 was never detected and the NOR of chromosome 10 was always detected in this pig's lymphocytes. Neither NOR could be detected in growing, subconfluent pig-mouse clones. This may mean that the NOR of chromosome 10 is deleted or concealed. Attempts to visualize the NOR by a treatment which has succeeded with other hybrids failed. Since the attempts were unsuccessful a firm interpretation cannot be given. NORs are detected by silver impregnation which is thought to depend on the non-histone proteins of the chromosome (Goodpasture and Bloom, 1975; Howell et al., 1975; Howell, 1977; Schwarzacher et al., 1978). These may be rich in accessible sulfhydryl and disulfide groups (Buys and Osinga, 1980). The intensity of silver impregnation reflects the intensity of transcription; inactive NORs are not detected (Miller et al., 1976a, 1976b, 1978). This relationship has been verified for the embryogenesis of the mouse (Engel et al., 1977; Hansmann et al., 1978), the spermatogenesis of various mammals (Schmid et al., 1977; Hofgartner et al., 1979), and the aging of humans (Buys et al., 1979). Although the NOR of pig chromosome 10 could not be detected in the hybrids, despite the clear identification of more than 100 No. 10 chromosomes, the mouse NORs were not affected. There were 8 to 13 mouse NORs per metaphase spread, in agreement with a study of human-mouse hybrids (Nielsen et al., 1979). Human-mouse hybrids which lose human chromosomes preferentially do not synthesize human 28S rRNA (Eleceiri and Green, 1969;

Marshall et al., 1975), and the human NORs do not react with silver nitrate (Miller et al., 1976a). Human-mouse hybrids which retain human chromosomes preferentially do synthesize human 28S rRNA (Croce et al., 1977), and the human NORs do react with silver nitrate (Miller et al., 1976b). Similar reports exist for rat-human hybrids (Tantravahi et al., 1979b). There are exceptions, e. g., rat-mouse hybrids may synthesize both kinds of rRNA (Kuter and Rodgers, 1975), although these hybrids lose rat chromosomes preferentially. Undetected human NORs and undetected mouse NORs of human-mouse hybrids can be visualized following treatment of the clones with SV-40 virus or TPA and this is accompanied by renewed synthesis of the appropriate 28S rRNA (Soprano et al., 1979; Soprano and Baserga, 1980). These reports demonstrate that the undetected NOR is retained, but is concealed by its inactivity or by something related to its inactivity. Presumably the same explanation applies to the non-detection of pig NORs in pig mouse hybrids.

The inactivity of NORs cannot be dismissed as an aberration due to unnatural conditions. The transcription of rRNA undergoes rate changes during gametogenesis, early development, and aging (see above). The cytoplasm of Xenopus blastomeres can inhibit rRNA synthesis (Shiokawa and Yamana, 1967). Only one member of a pair of large acrocentric chromosomes of the guinea pig has an NOR; the other chromosome is heavily condensed (Ohno et al., 1961). Interspecific hybrids of plants, produced by cross-fertilization, express the NORs from the parent whose chromosomes are retained and this can be reversed by backcrossing (Keep, 1962). This evidence of continuing modulation has led to the suggestion of a reversible suppression by methylation of DNA (Tantravahi et al., 1979a).

Pig G-6PD, HPRT, and GLA are syntenic and map to the X-chromosome, extending Ohno's hypothesis of X-chromosome conservation (Ohno, 1973) to yet another mammalian species. There is no known exception to Ohno's hypothesis that the genetic content of the X-chromosome is identical in all mammals, marsupial and placental. The loci for GLA, G-6PD, HPRT, and PGK have been assigned to the X-chromosome in 15 mammalian species (Pearson and Roderick, 1979), e. g., humans (Grzeschik et al., 1972a, 1972b; Ricciuti and Ruddle, 1973; Kozak et al., 1975; Shows and Brown, 1975; Chapman and Shows, 1976), the gorilla (Garver et al., 1978), and cattle (Heuertz and Hors-Cayla, 1978). Two, or three, of these loci have been assigned to the X-chromosome of the Chinese hamster (Westerveld et al., 1972), the horse (Deys, 1972), the Indian muntjac (Shows et al., 1976), and marsupials (Graves et al., 1979). For humans, PRPP synthetase has been assigned to the X-chromosome (Yen et al., 1978; Becker et al., 1979) and OTC is X-linked (Ricciuti et al., 1976). It would be particularly interesting to learn the location of the synthetase because of the utilization of PRPP by HPRT. The HPRT locus is located on the long arm of the human X-chromosome, separated from the centromere by the GLA locus (Francke and Taggart, 1980). The order is reversed in the mouse; centromere, HPRT, and GLA (Francke and Taggart, 1980). The locus for mouse PGK also lies between the centromere and GLA (Lusis and West, 1976; Nielsen and Chapman, 1977). Ohno's hypothesis has been verified, but it is not yet clear that the order of sex-linked genes is conserved. If the order of sex-linked genes is related to the banding pattern we should expect to find some conservation of the sequence. There are similar, perhaps homologous, patterns in all X-chromosomes despite great differences in size and overall morphology (Grouchey et al., 1972; Turleau et al., 1972; Borrow and Madan, 1973; Evans et al., 1973; Lejeune et al., 1973; Yosida and Sagai, 1973; Buckland and Evans, 1978a, 1978b). The banding pattern of the X-chromosome of humans is almost identical with that of the chimpanzee (Lin et al., 1973; Yunis et al., 1980). The X-chromosomes of 60 mammalian species have two trypsin-resistant bands despite gross morphological differences (Pathak and Stock, 1974). I found two trypsin-resistant bands in the X-chromosome of the pig. There is no reason to assume that the evidence for concordance of genetic clusters and banding patterns, within the X-chromosome, is exhausted.

The assignment of SOD-1 to pig chromosome 9 is partly fortuitous. The necessary reagents were available as components of other tests and it was fortunate that the initial tests matched up clones that gave cleancut evidence of selective retention and loss. There is only one chromosome whose retention and loss match that of the enzyme, chromosome 9. The identification of the activity as due to SOD-1, not SOD-2, is as certain as the identifications made in prior assignments of SOD enzymes. The identification could be improved by use of unambiguous techniques for distinguishing SOD-1 from SOD-2. The SOD enzymes of the pig (Widar et al., 1975) and other mammals (Bauer and Schorr, 1969; Utter, 1971; Burnet, 1972) may occur in multiple forms. Multiplicity may be isozymic (Weisiger and Fridovich, 1973) or allelic (Beckman, 1973; Beckman and Beckman, 1975; Beckman et al., 1975). The fact that the enzyme is a dimer means that it can probably form mixed dimers with isozymic variants, and there is ample evidence that this is a general phenomenon. Similarly, pig SOD-1 formed heteropolymers with mouse SOD-1. Heteropolymerization is, in fact, the only experimental observation to prove that the pig enzyme is SOD-1; the other evidence is derived from comparisons with previous reports.

SOD-1 and SOD-2 are not isozymic in the original meaning of the terms "isozyme" and "isoenzyme" SOD-1 is a Cu++ and Zn++ metalloenzyme of remarkable thermal stability and amino acid content which forms dimers, and mimics the configuration of immunoglobulin domains. SOD-2 is a Mn++ metalloenzyme which forms tetramers, and is unusual only for its location in the mitochondrion. It is not surprising that the two enzymes do not combine with each other to form heteropolymers; they could hardly be more different (Beckman and Holm, 1975). The catalysis of the dismutation of superoxide anion may be misleading. One of these enzymes may react with other species of active oxygen, e. g., singlet oxygen. SOD-1 is elevated in trisomic 21 humans (Sichitiu et al., 1974; Feaster et al., 1977), the first indication that it is carried on human chromosome 21 to which it has been assigned (Tan et al., 1973; Moore et al., 1977). It is syntenic with the interferon receptor (IfRec, formerly AVG or AVP) (Revel et al., 1976; Sinet et al., 1976) and glycinamide ribonucleotide synthetase (Moore et al., 1977). SOD-1 and IfRec have been assigned to mouse chromosome 16 (Cox et al., 1980). If the SOD genes are components of conserved genetic complexes we might find the SOD-2 gene on mouse chromosome 17. Mouse chromosome 17 carries the major histocompatibility complex, and SOD-2 is syntenic with the homologous complex carried on human chromosome 6 (Creagan et al., 1973; Van Someren et al., 1974). The assignment of SOD-1 to pig chromosome 9, if verified, is a first step in establishing synteny groups and making chromosome assignments. This should proceed rapidly because of the vigorous growth of pig-mouse hybrids and the cleancut contrasts which distinguish pig from mouse chromosomes. The value of the pig for work of this kind is the availability, numbers, and controlled variety of the species.

It has been postulated that tetraploidization occurred 2-3 X 10^8 years ago, doubling the chromosome number and thereby permitting major rearrangements of gene groupings. Rearrangements may have been conserved by accidents which usually leave ancestral linkage groups intact, e. g., Robertsonian fusions, inversions, and gene duplications (Comings, 1972; Ohno, 1973). This hypothesis predicts detection of the same linkage groups in different species. Some of the genes that are syntenic in humans are also syntenic in the chimpanzee, gorilla, orangutang, and baboon, and in the African green, capuchin, and rhesus monkeys (Pearson and Roderick, 1979). These synteny groups are located in chromosomes judged, by banding, to be homologous with human chromosomes (Pearson and Roderick, 1979; Yunis et al., 1980). TK and galactokinase (GALK) are syntenic in humans and the chimpanzee, and this synteny is also found in the Chinese hamster, the mouse, and other non-primates

(Kozak et al., 1977; Pearson and Roderick, 1979). The group-specific component (Gc), of the alpha-2-globulin which binds vitamin-D, is closely linked to serum albumin in humans (Weitkamp et al., 1966) and the horse (Weitkamp and Allen, 1979). The mapping of mouse genes (Davisson and Roderick, 1980; Womack, 1980) has been accelerated by analysis of clones of hybrid cells (Francke et al., 1977; Lalley et al., 1978a, 1978b; Francke and Taggart, 1979, 1980). Five enzymes (Ak-2, Eno-1, Gpd-1, Pgd, and Pgm-2; see pages xiv to xvi) are syntenic in the mouse (Hutton and Frederick, 1970; Chapman, 1975; Lalley et al., 1978a) and in the human (Pearson and Roderick, 1979). All are assigned to mouse chromosome 4, and all but Gpd-1 are assigned to the short arm of human chromosome 1. It will be useful to learn which of the synteny groups of humans and the mouse are conserved in the pig. More directly, does pig chromosome 9 have a gene cluster like that in human chromosome 21 and mouse chromosome 16, and which pig chromosomes correspond in this respect to human 1 and mouse 4, and to human 6 and mouse 17?

Interspecific somatic cell hybrids are powerful genetic tools. It is possible to hybridize cells of many mammalian species to produce permanent clones which lose the chromosomes of one species while retaining the chromosomes of the other. Within a clone it is possible to distinguish many of the homologous enzymes (Khan, 1971; Nichols and

Ruddle, 1973; Harris and Hopkinson, 1976), prove the homology of two enzymes, prove the loss of one homologue and the retention of the other, contrast the loss and retention of particular chromosomes, and prove the concordance of enzyme loss and chromosome loss. This is assisted by the distinctive banding of the different chromosomes of a species, and by the recurrence of similar banding patterns in chromosomes of different species. If the banding pattern of a chromosome is determined by the base sequences of its genes, the banding pattern may pinpoint regions which are homologous with those of other species. We may be able to anticipate many gene assignments from the concordance of some banding patterns and gene assignments (Turleau et al., 1972; Finaz et al., 1973; Ruddle and Creagan, 1975; Minna et al., 1976). Interspecific recurrences of banding patterns and synteny groups are clear evidence of selection to maintain chromosome regions and clusters of genetic loci, but it is the exceptions to conservation and the rearrangement of loci within clusters which may prove most instructive. We do not yet have the information with which to assess the frequency and meaning of conservation, the exceptions, and rearrangement within clusters. Banding patterns may speed the acquisition of the necessary information as they have facilitated the identification of chromosomes and detected their alteration by deletion, linear duplication, fusion, inversion, and translocation.

I searched 140 G-banded metaphase spreads of pig-mouse clones for altered pig chromosomes, and for pig-mouse translocations, without success; the pig chromosomes remain intact and separate. There is no obvious reason to believe that the non-detection of pig NORs in pig-mouse clones represents the deletion of the NOR from chromosome 10. Nor did I find any reason to think that the assignments of enzymes to the X-chromosome, or chromosome 9, are wrong by chance of chromosome deletion or translocation. The possibility of a minute alteration which might compromise these assignments cannot be excluded, but is deemed unlikely because it, or its equivalent, would have had to occur in different clones. If minute alterations are as random as major alterations, the recurrence of the same or equivalent minute alteration would be an interesting phenomenon by itself, more important than the assignments I have made. Six genes or gene complexes have been assigned to pig chromosomes: one NOR to chromosome 8, one NOR to chromosome 10, three enzymes to the X-chromosome, and one enzyme to chromosome 9 (Fig. 25). The homogeneity, or heterogeneity, of five of the 11 clones used to make the assignment to chromosome 9 was shown to be conventional (Fig. 14, Table 5). The six assignments made here are the first assignments to pig chromosomes.

SUMMARY

1. Hybrids of pig and mouse cells were obtained by fusion of male pig lymphocytes with mouse cells from an established line (RAG) deficient in hypoxanthine-guanosine phosphroribosyltransferase (HPRT-).

2. The hybrids grew rapidly. Thirty permanent clones were established and examined in detail. The clones lost pig chromosomes preferentially. The loss was greatest for the non-acrocentric chromosomes, 1 to 12. Acrocentric chromosome 16 was identified in all clones in which it was sought (28).

3. NORs were detected in chromosomes 8 and 10 of pig lymphocytes; the NOR of chromosome 10 was detected in all parental pig lymphocytes. The NOR of pig chromosome 10 could not be detected in pig-mouse hybrids although the number of detectable mouse NORs was not reduced. Four clones were examined in detail; 111 No. 10 chromosomes were NOR- and none were NOR+. An attempt to overcome this by treatment of the cells with TPA did not succeed.

4. Pig alpha galactosidase (GLA), HPRT, and glucose-6-phosphate dehydrogenase (G-6PD) are syntenic and are assigned to the X-chromosome: seven concordant clones, five positive and two negative ($p < 0.05$). This agrees with the findings for other species.

5. Dimeric superoxide dismutase (SOD-1) is assigned to pig chromosome 9: 11 concordant clones, seven positive and four negative ($p < 0.01$). This is the first assignment of an enzyme to an autosome of a domestic or agricultural animal by direct identification of the chromosome.

Tablela. The Distribution of Pig Chromosomes Among 26 Pig-mouse Hybrid Clones.[1]

Hybrid	Pig Chromosome Constitution:																		
Clone:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	X	Y
PLR 26	-	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-
PLR 13	+	-	-	-	-	+	-	+	+	+	-	-	+	+	+	+	+	+	+	-
PLR 71	-	+	-	+	+	-	+	+	-	-	+	+	+	-	-	+	+	+	+	-
PLR 22	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
PLR 76	-	+	-	+	+	-	-	-	-	-	+	-	+	+	-	+	+	+	+	-
PLR 9	+	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	+	+	+	-
PLR 96	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
PLR 50	-	+	-	+	-	-	-	+	+	+	-	-	+	+	+	+	-	-	+	-
PLR 16	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
PLR 1	-	-	+	-	-	+	-	+	-	+	+	-	-	-	+	+	+	-	+	-
PLR 20	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	-
PLR 3	-	-	-	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+
PLR 2	+	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+
PLR 5	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-	+	-	+
PLR 7	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
PLR 28	+	+	-	+	+	-	-	+	-	+	+	-	+	-	+	+	-	-	+	+
PLR 23	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	+	-
PLR 4	+	+	-	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	-	-
PLR 75	+	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-
PLR 95	-	-	-	+	-	+	+	-	+	-	+	-	+	-	+	+	+	+	+	-
PLR 81	-	+	-	+	+	+	-	+	-	+	+	-	+	-	-	+	+	-	+	-
PLR 85	-	+	-	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	+	-
PLR 19	-	-	-	+	-	-	+	+	+	+	+	-	+	-	+	+	+	+	-	-
PLR 22	-	-	-	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	-	+
PLR 52	-	+	-	+	-	+	+	+	+	-	+	-	+	-	+	+	+	+	-	-
PLR 5[illegible]	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-

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[1] A given pig chromosome was scored as present (+) if it was detected in 20% or greater of the cells analyzed. The number of metaphase cells analyzed ranged from 14-22 for each clone.

Table 1b. Segregation of Pig Chromosomes and X-linked Enzymes Among Seven Pig-mouse Hybrid Clones.[1]

Hybrid Clone	Pig Chromosome Constitution																				Expression of Pig Enzymes:		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	X	Y	GLA	HPRT	G6PD
PLR 1	-	+	-	+	+	-	-	+	+	+	+	-	+	-	+	+	-	-	+	-	+	+	+
PLR 2	+	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+
PLR 3	-	-	-	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
PLR 4	+	+	-	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	-	-	-	-	-
PLR 5	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-
PLR 6	-	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+
PLR 7	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+

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[1] Segregation of pig chromosomes and enzyme markers GLA, HPRT, and G6PD in pig-mouse somatic cell hybrids. Enzyme analyses and karyotyping were performed on the same passage of cells. A clone was scored positive if a chromosome was present in 20% or greater of the cells analyzed. The number of metaphase spreads analyzed ranged from 14-22 for each clone.

Table 2. Distribution of NOR's Among Donor Pig Lymphocytes.

Donor Pig	No. of Cells Scored	No. of Copies of Chromosomes/cell With NOR$^+$ Sites on Chromosomes	
		No. 8	No. 10
Pig #2	23	0	2
Pig #3	14	1	2
	4	2	1
*Pig #4	24	0	2
Pig #5	16	0	2
Pig #6	20	0	2

*Lymphocytes from Pig #4 were used as parental cells in the production of pig-mouse somatic cell hybrids.

Table 3. Distribution of NOR's in Pig-mouse Somatic Cell Hybrids.

Hybrid Clone	No. of Cells Scored	Average No. of RAG Chromosomes/cell With NOR$^+$ Sites	Total No. of Copies of Pig Chromosome No. 10 Observed without NOR$^+$ Sites
PLR 52	20	13	30/30
PLR 20	22	8	26/26
PLR 8	24	10	31/31
PLR 11	21	9	24/24

Table 4. Distribution of NOR's Among Pig-mouse Somatic Cell Hybrids After TPA Treatment.

Hybrid Clone	Average No. of RAG Chromosomes/Cell NOR-positive Sites	Total No. of Copies of Pig Chromosome No. 10 Observed Without NOR-positive Sites After Treatment:		
		Control (no TPA)	100 nM TPA	200 nM TPA
PLR 8	9	32/32	37/37	35/35
PLR 11	8	27/27	29/29	30/30
PLR 20	8	23/23	28/28	30/30
PLR 52	13	25/25	28/28	29/29

Table 5. Segregation of Pig Chromosomes and Superoxide Dismutase (SOD-1) Among Eleven Pig-mouse Hybrid Clones.[1]

Hybrid Clone	Pig Chromosome Constitution																				Expression of pig
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	X	Y	SOD-1
PLR 26	-	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
PLR 13	+	-	-	-	-	+	-	+	+	+	-	-	+	+	+	+	+	+	+	-	+
PLR 71	-	+	-	+	+	-	+	+	-	-	+	+	+	-	-	+	+	+	+	-	-
PLR 22	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
PLR 76	-	+	-	+	+	-	-	-	-	-	+	-	+	+	-	+	+	+	+	-	-
PLR 9	+	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	+	+	+	-	+
PLR 96	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
PLR 50	-	+	-	+	-	-	-	+	+	+	-	-	+	+	+	+	-	-	+	-	+
PLR 16	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+
PLR 31	-	-	+	-	-	+	-	+	-	+	+	-	-	-	+	+	+	-	+	-	-
PLR 20	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	-	+

---

[1] Segregation of pig chromosomes and superoxide dismutase in pig-mouse somatic cell hybrids. Enzyme analyses and karyotyping were performed on the same passage of cells. A clone was scored positive if a chromosome was present in 20% or greater of the cells analyzed. The number of metaphase spreads analyzed ranged from 15-35 for each clone.

Table 4. Chi-Square Test on the Assignment of Superoxide Dismutase Gene (SOD-1) to Pig Chromosome No. 9.

No. of Clones With (+)/Without (-) Pig Chromosome No. 9 :	No. of Clones With (+)/Without (-) Pig SOD-1 Activity : +	-	Totals :
+	7	0	7
-	0	4	4
Totals :	7	4	11

$\chi^2 = 11.0$ $P < 0.005$ Degrees of Freedom = 1

$\chi^2_{\text{(with Yates correction)}} = 7.10$ $P < 0.01$ Degrees of Freedom = 1

V

Figure 1. Steps in purine and pyrimidine biosynthesis basic to the selection of hybrids by use of aminopterin or alanosine (after Grzeschik, 1973).

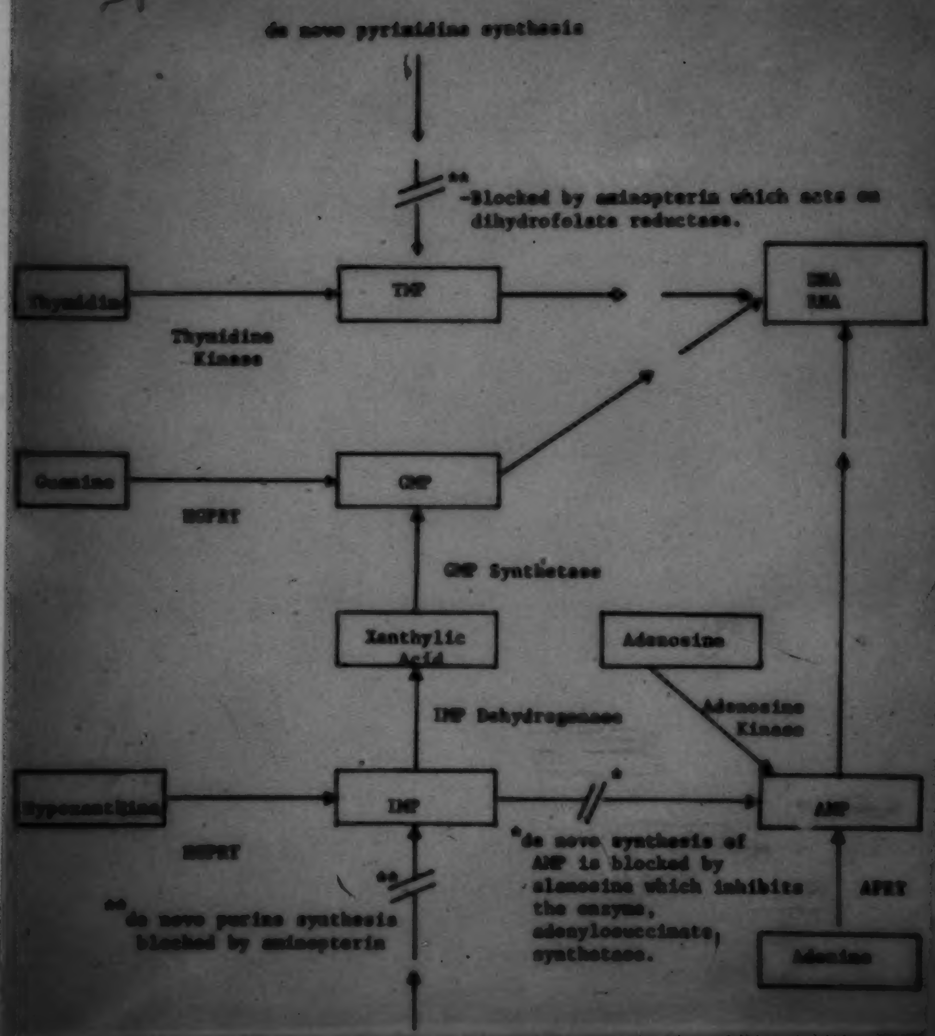

de novo pyrimidine synthesis
**
-Blocked by aminopterin which acts on dihydrofolate reductase.
Thymidine
TMP
DNA
RNA
Thymidine Kinase
Guanine
GMP
HGPRT
GMP Synthetase
Xanthylic Acid
Adenosine
IMP Dehydrogenase
Adenosine Kinase
Hypoxanthine
IMP
*
AMP
HGPRT
*de novo synthesis of AMP is blocked by alanosine which inhibits the enzyme, adenylosuccinate, synthetase.
APRT
**
**de novo purine synthesis blocked by aminopterin
Adenine

Figure 2. Linkage groups of the domestic pig (Sus scrofa domesticus)(after Andresen, 1966a, 1966b, 1971; Imlah, 1965).

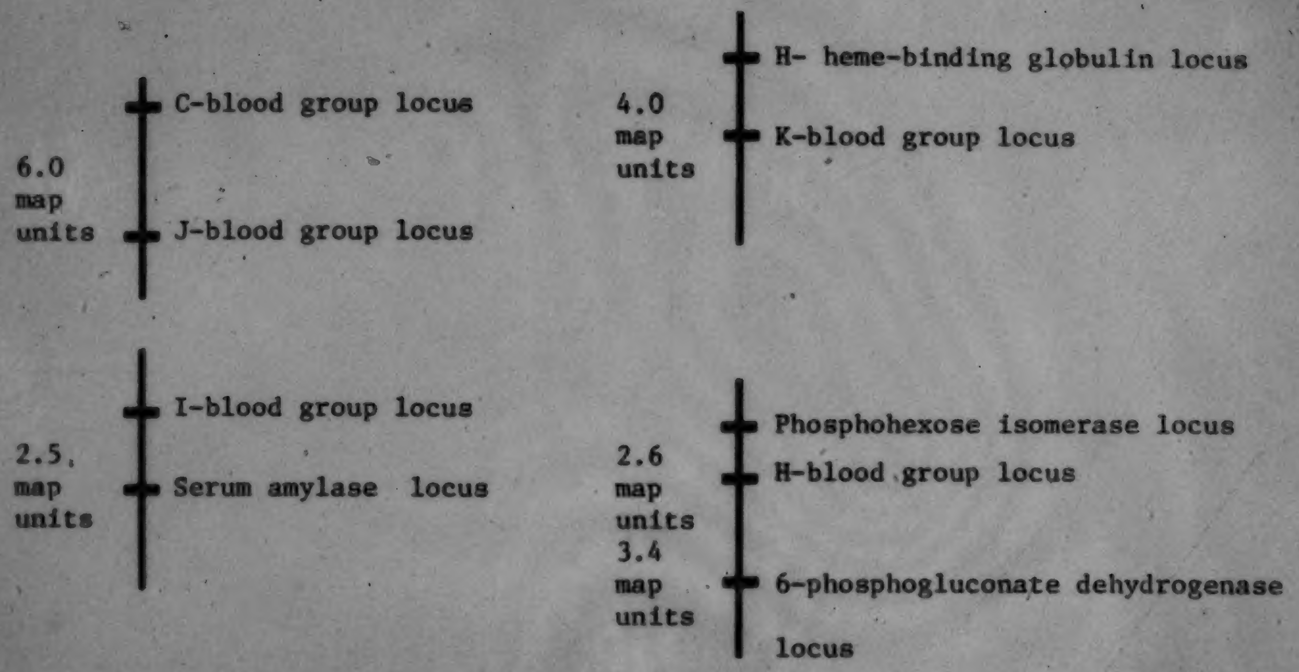

C-blood group locus
6.0
map
units
J-blood group locus
H- heme-binding globulin locus
4.0
map
units
K-blood group locus
I-blood group locus
2.5
map
units
Serum amylase locus
Phosphohexose isomerase locus
2.6
map
units
H-blood group locus
3.4
map
units
6-phosphogluconate dehydrogenase
locus

Figure 3. A G-banded karyotype of a parental pig lymphocyte. The pig chromosomes are arranged and identified in accordance with standards (Lin et al., 1980; Reading Conference, 1980).

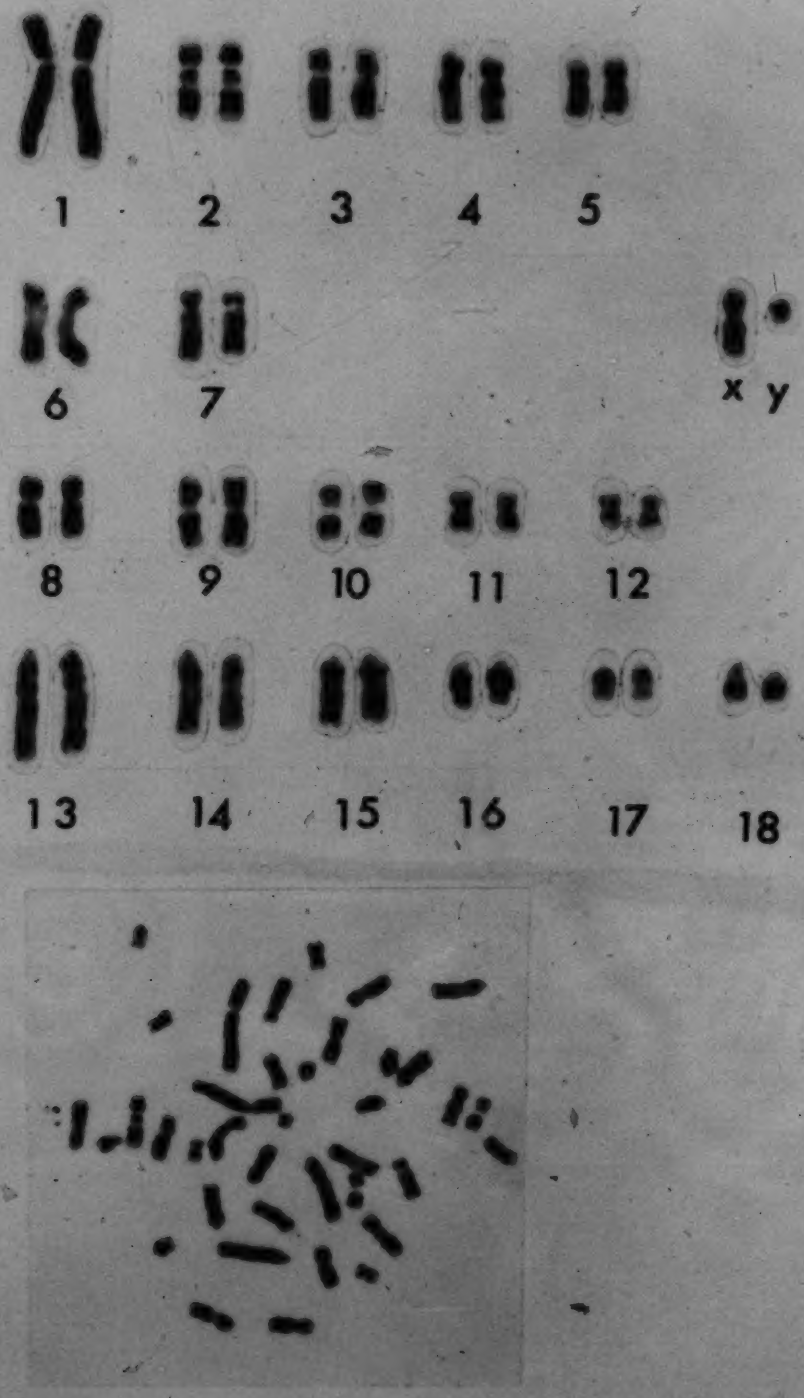

1
2
3
4
5
6
7
x
y
8
9
10
11
12
13
14
15
16
17
18

Figure 4. A Q-banded (actinomycin-D and Hoechst 33258) karyotype of a parental pig lymphocyte.

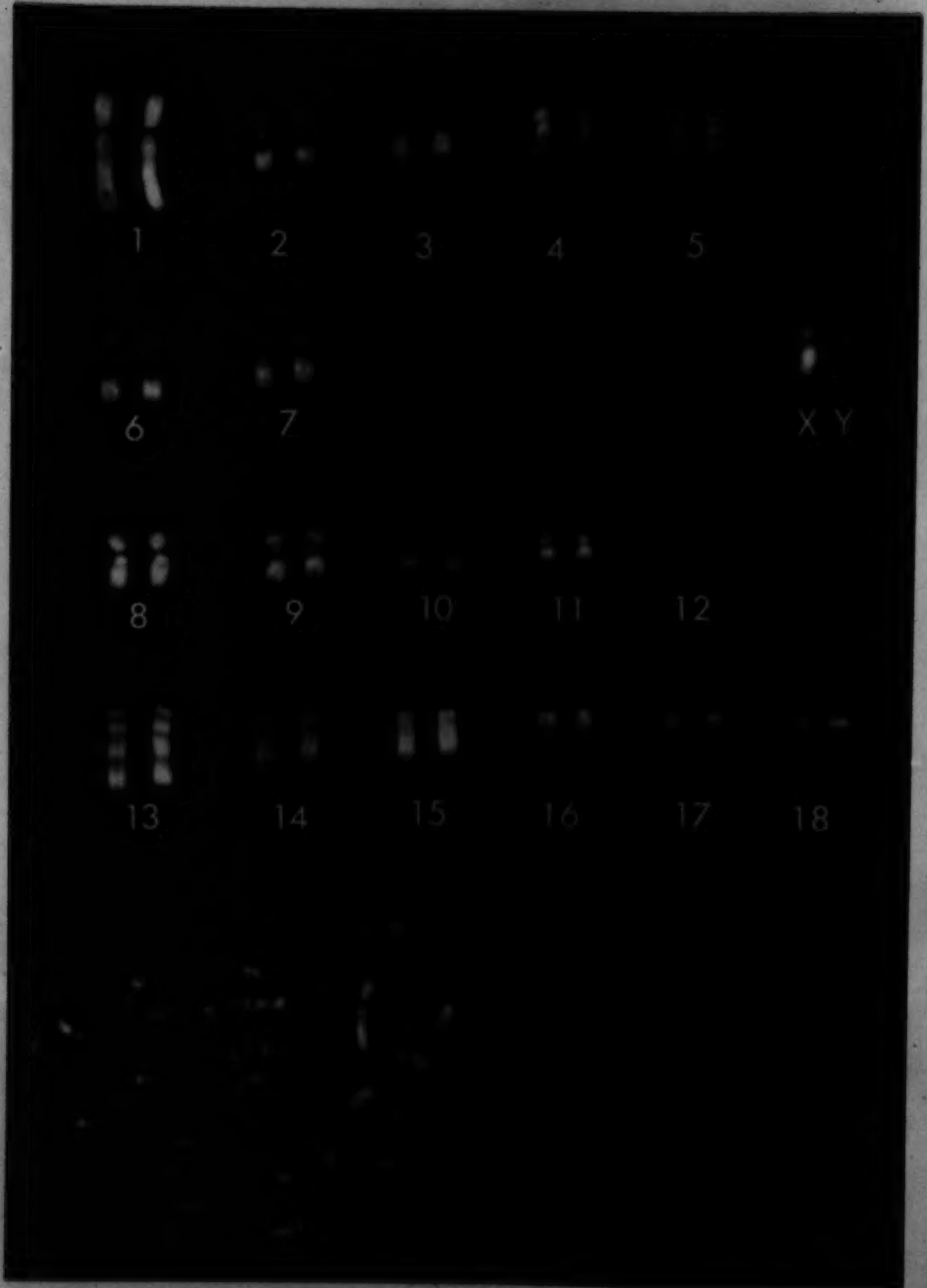

1
2
3
4
5
6
7
X Y
8
9
10
11
12
13
14
15
16
17
18

Figure 5. A G-banded karyotype of a mouse bone marrow cell. The mouse chromosomes are arranged and identified in accordance with standards (Committee on Standardized Genetic Nomenclature For Mice, 1972).

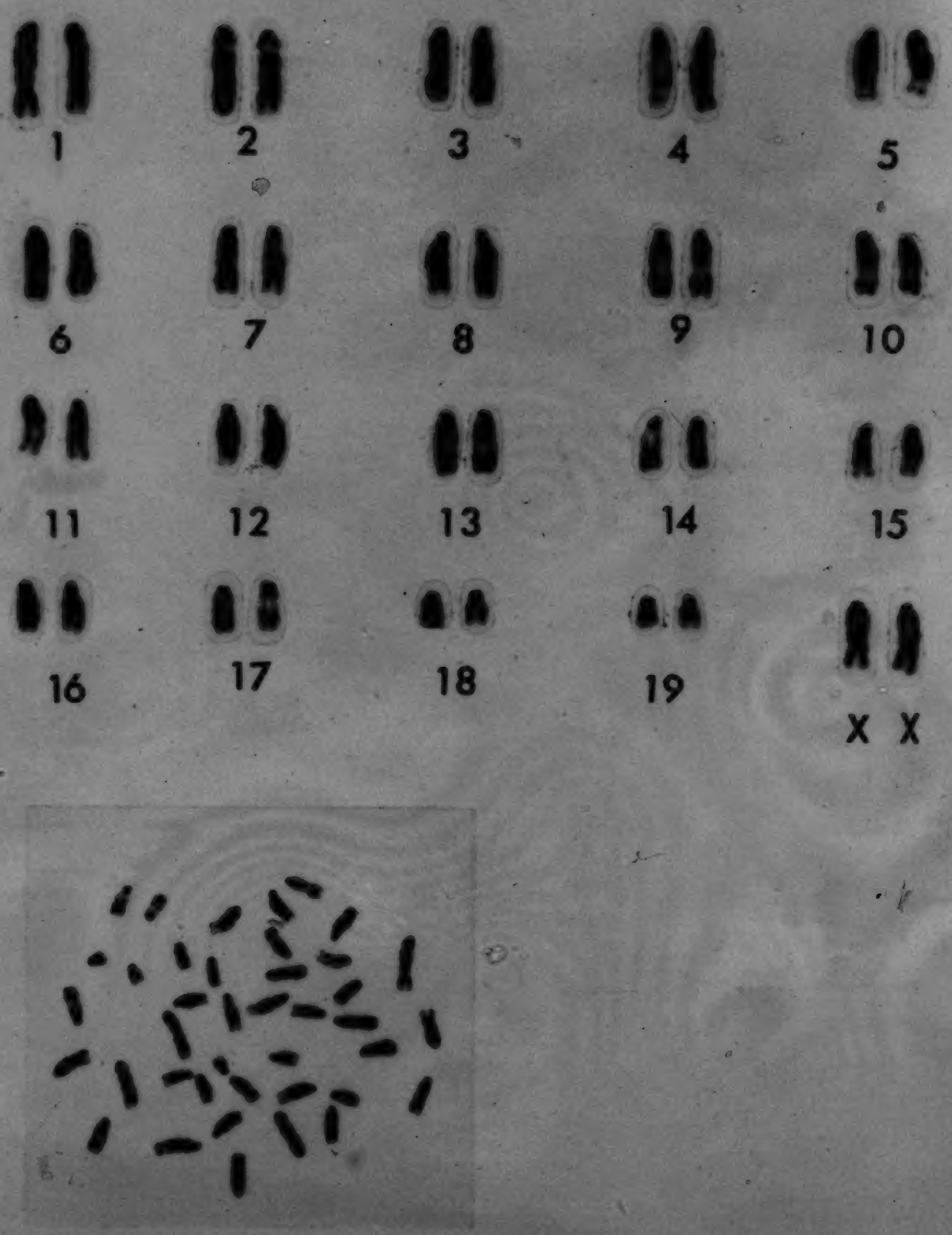

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X X

Figure 6. A G-banded karyotype of a parental RAG cell. The marker chromosomes are identified in accordance with a standard (Hashmi et al., 1974).

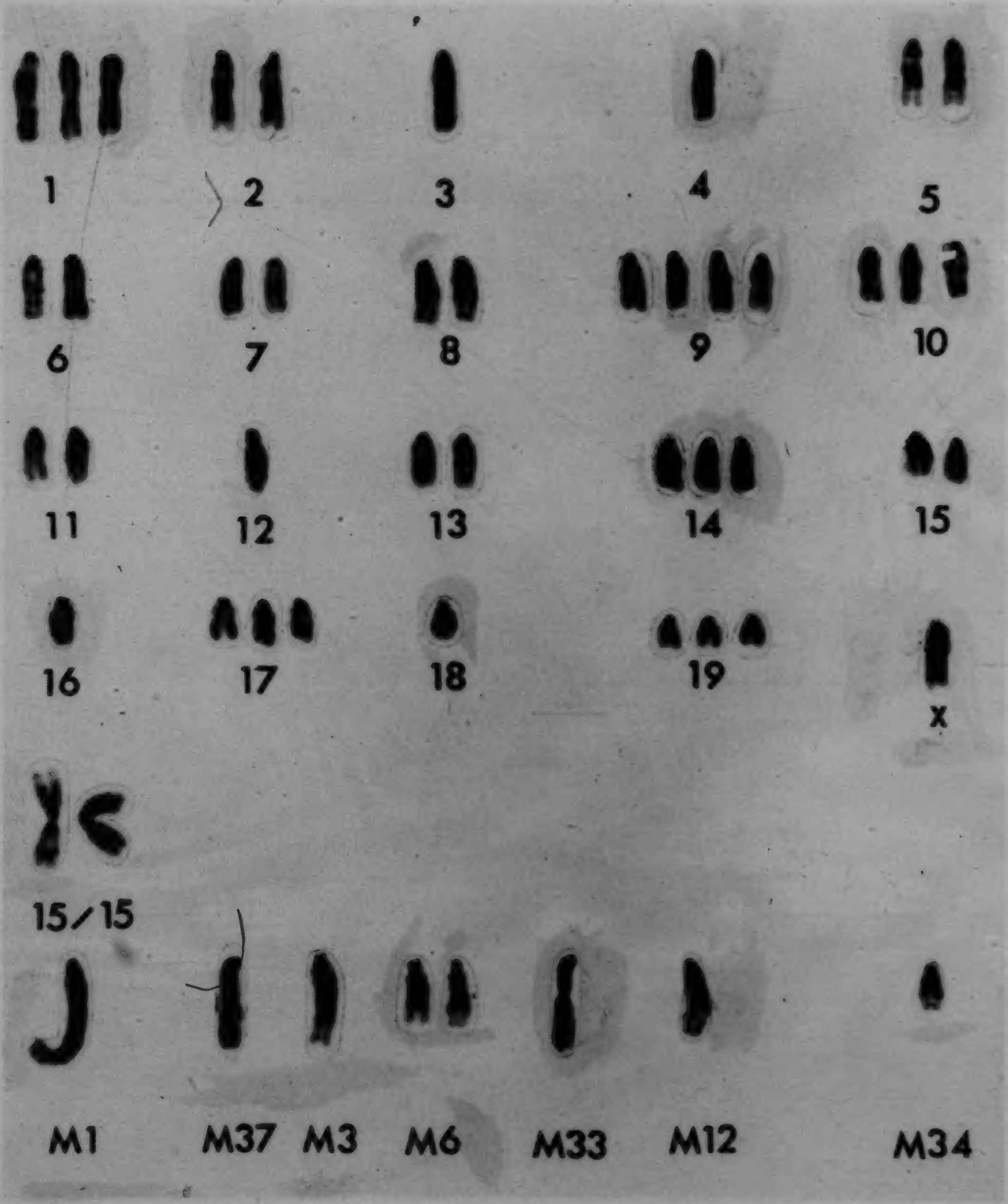

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
15/15
M1
M37
M3
M6
M33
M12
M34

Figure 7. A Q-banded (actinomycin-D and Hoechst 33258) karyotype of a parental RAG cell.

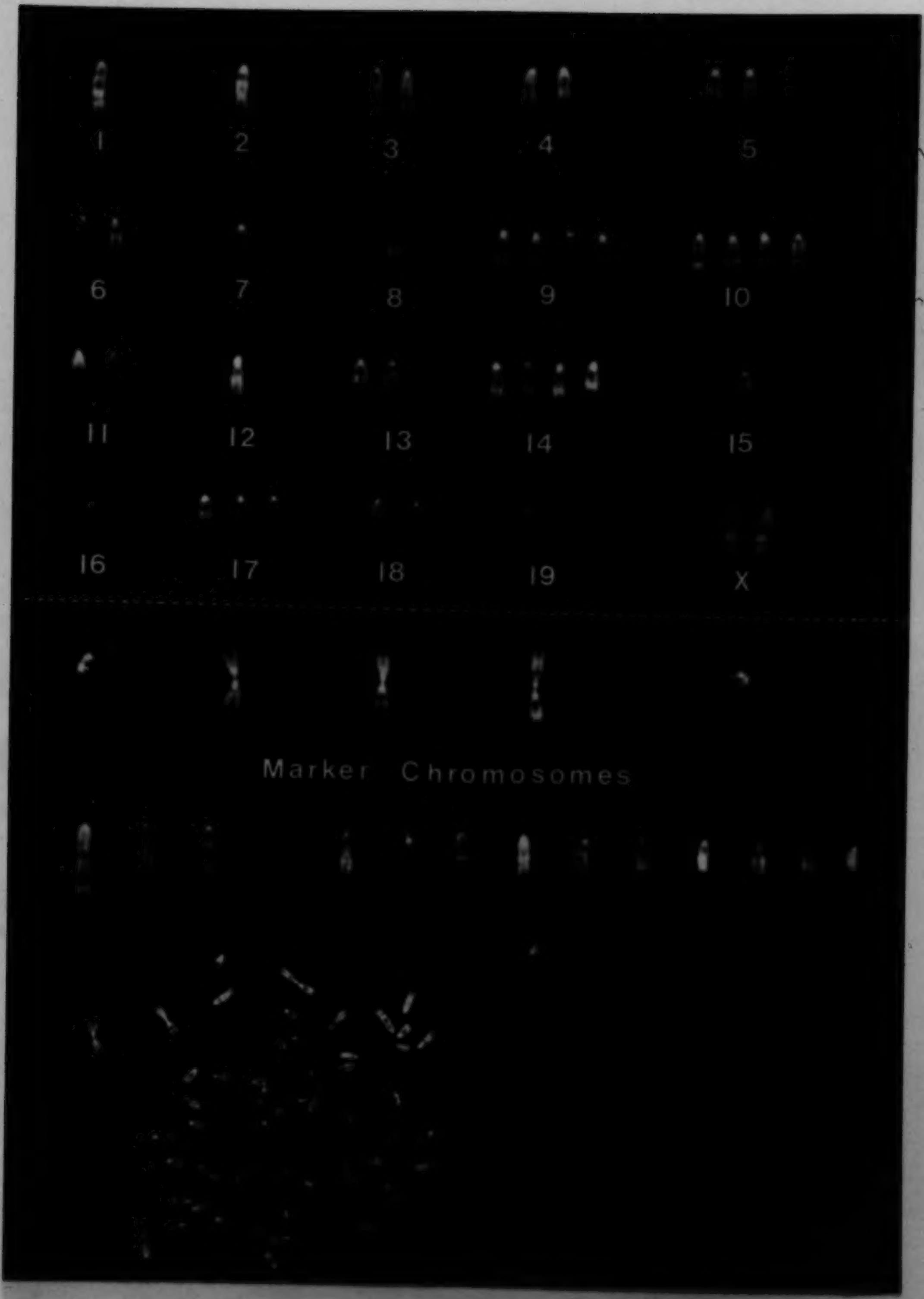

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
Marker Chromosomes

Figure 8. A G-banded karyotype of a pig-mouse hybrid cell.

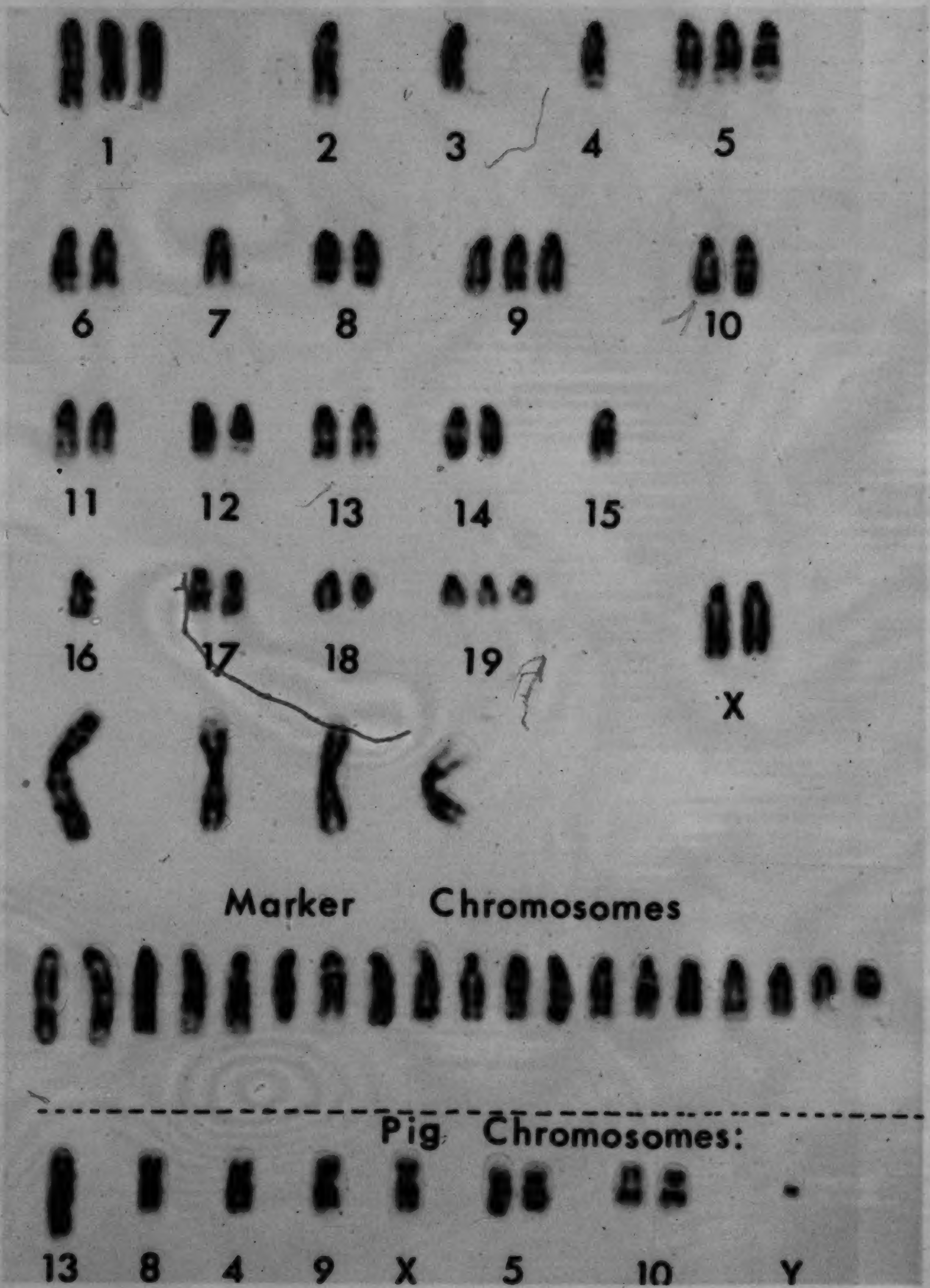

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
Marker Chromosomes
Pig Chromosomes:
13
8
4
9
X
5
10
Y

Figure 9. A Q-banded (actinomycin-D and Hoechst 33258) karyotype of a pig-mouse hybrid cell.

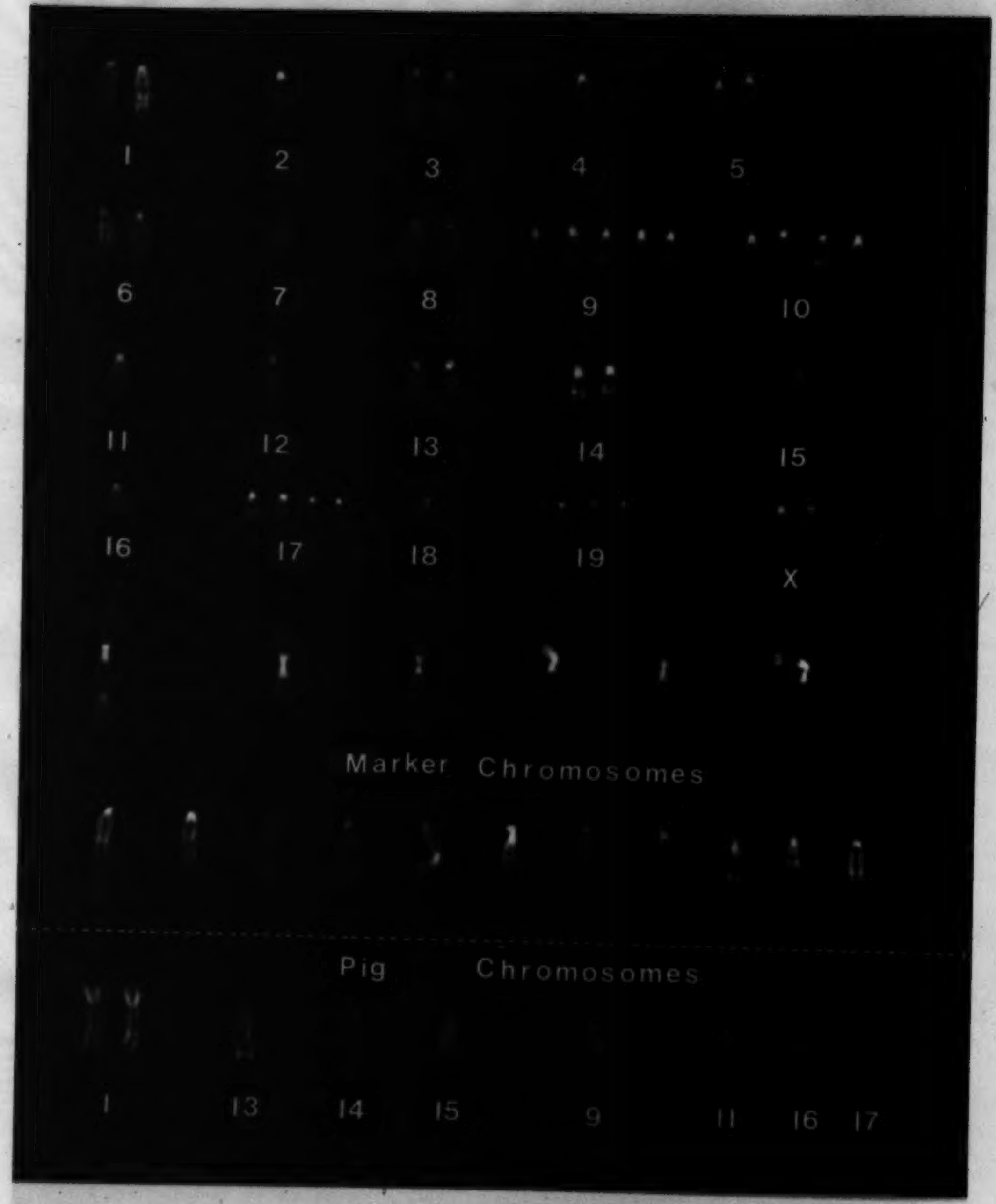
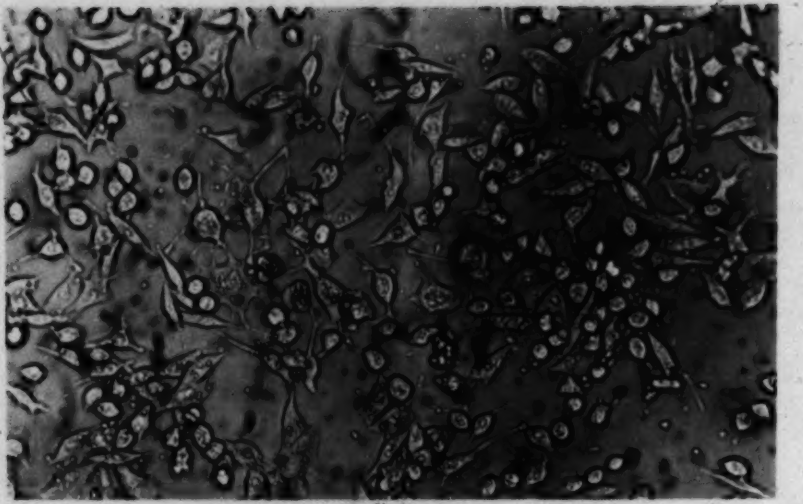
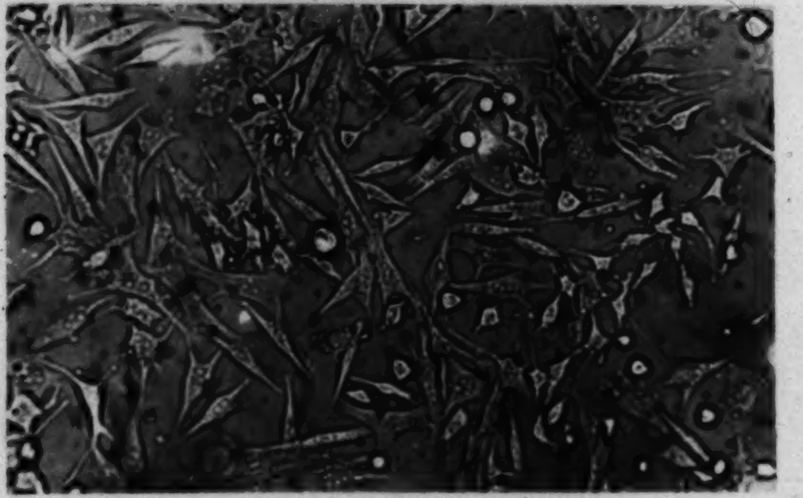

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
Marker Chromosomes
Pig Chromosomes
1
13
14
15
9
11
16
17

Figure 10. Photomicrographs of RAG and pig-mouse hybrid cells in culture: (a) parental RAG cells, and (b) pig-mouse hybrid cells.



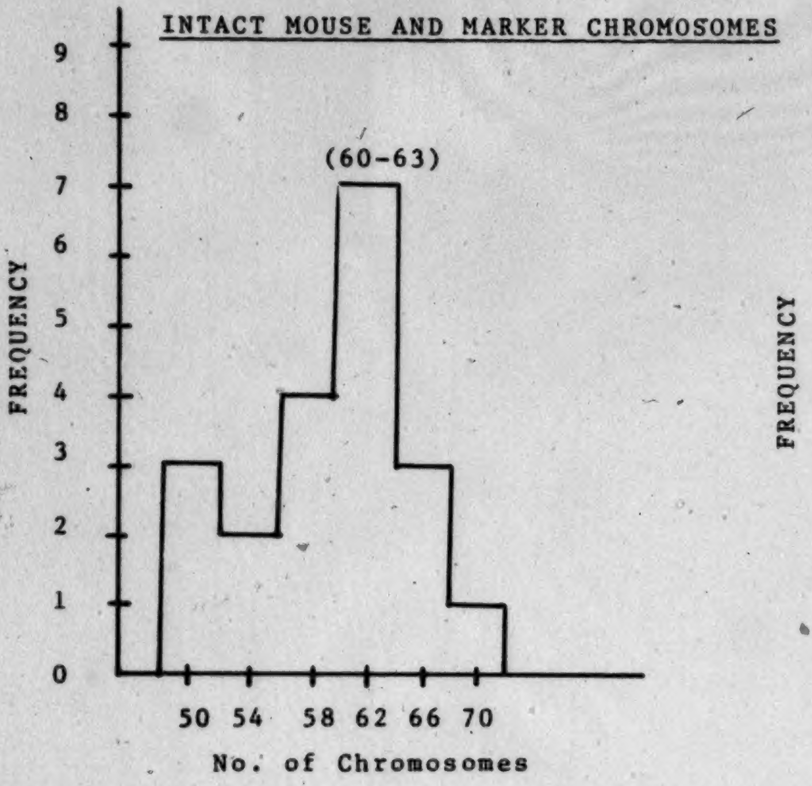
(a)



(b)

Figure 11. Frequency histograms of intact and marker chromosomes of parental RAG cells. The histograms represent 20 G-banded metaphase spreads. The frequencies are the numbers of metaphase spreads in the various classes; the class interval is 4, and the numbers on the X-axis are the midpoints plus 0.5.

CHROMOSOME DISTRIBUTIONS OF THE RAG CELL LINE


INTACT MOUSE AND MARKER CHROMOSOMES

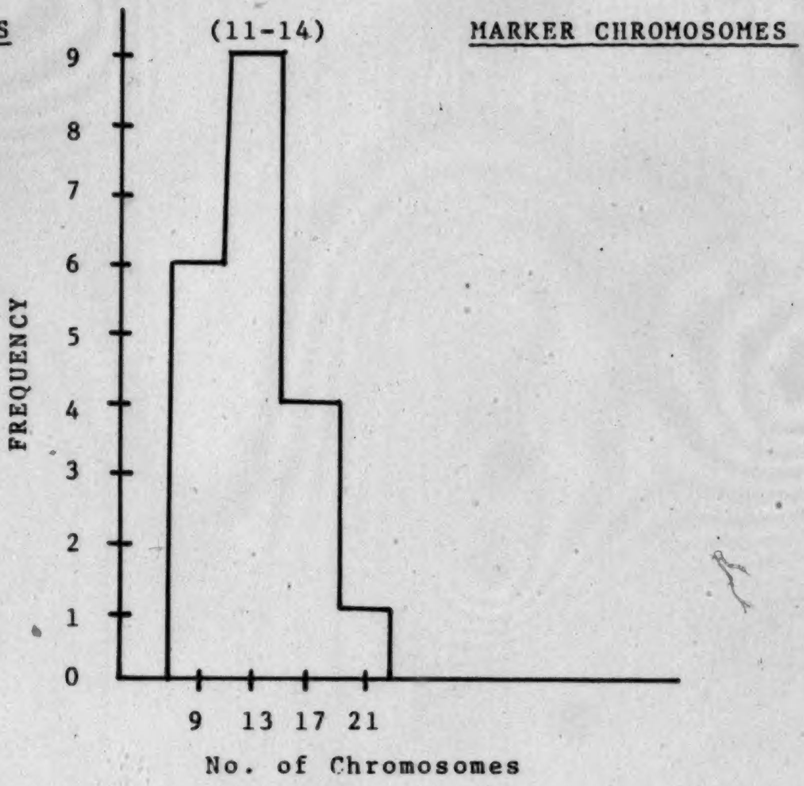
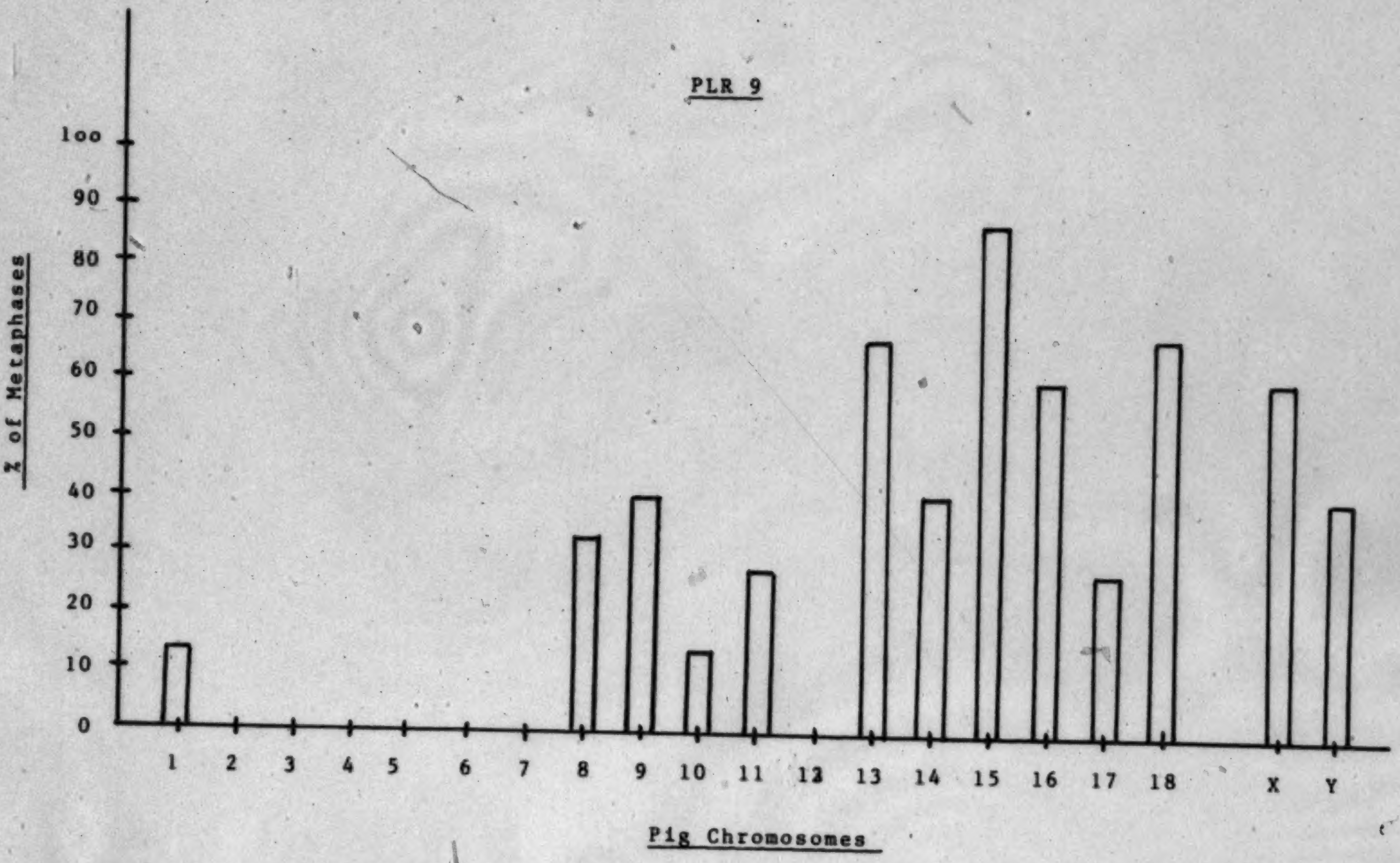

MARKER CHROMOSOMES

Figure 12. Distribution of pig chromosomes in pig-mouse hybrid clone PLR 9. The distribution represents 17 G-banded metaphase spreads. The number of metaphase spreads which retain at least one copy of a particular chromosome (X axis) is given in percent (Y axis).



PLR 9

100
90
80
70
60
50
40
30
20
10
0
% of Metaphases
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
X
Y
Pig Chromosomes

Figure 13. Distribution of pig chromosomes in pig-mouse hybrid clone PLR 8.

PLR 8

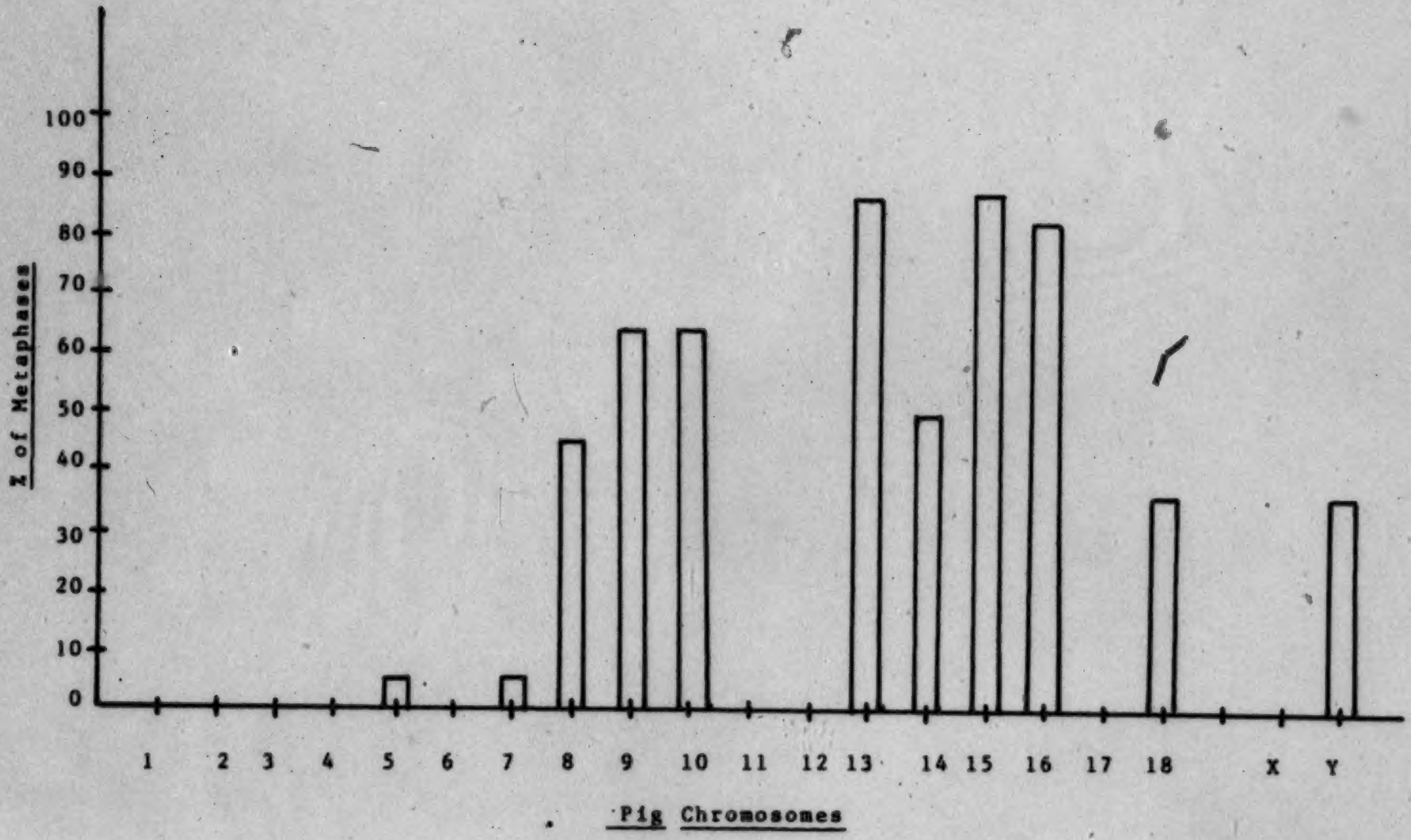

% of Metaphases
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
X
Y
Pig Chromosomes

Figure 14. Heterogeneity curves for five pig-mouse hybrid clones (see text, pp. 30 to 32).

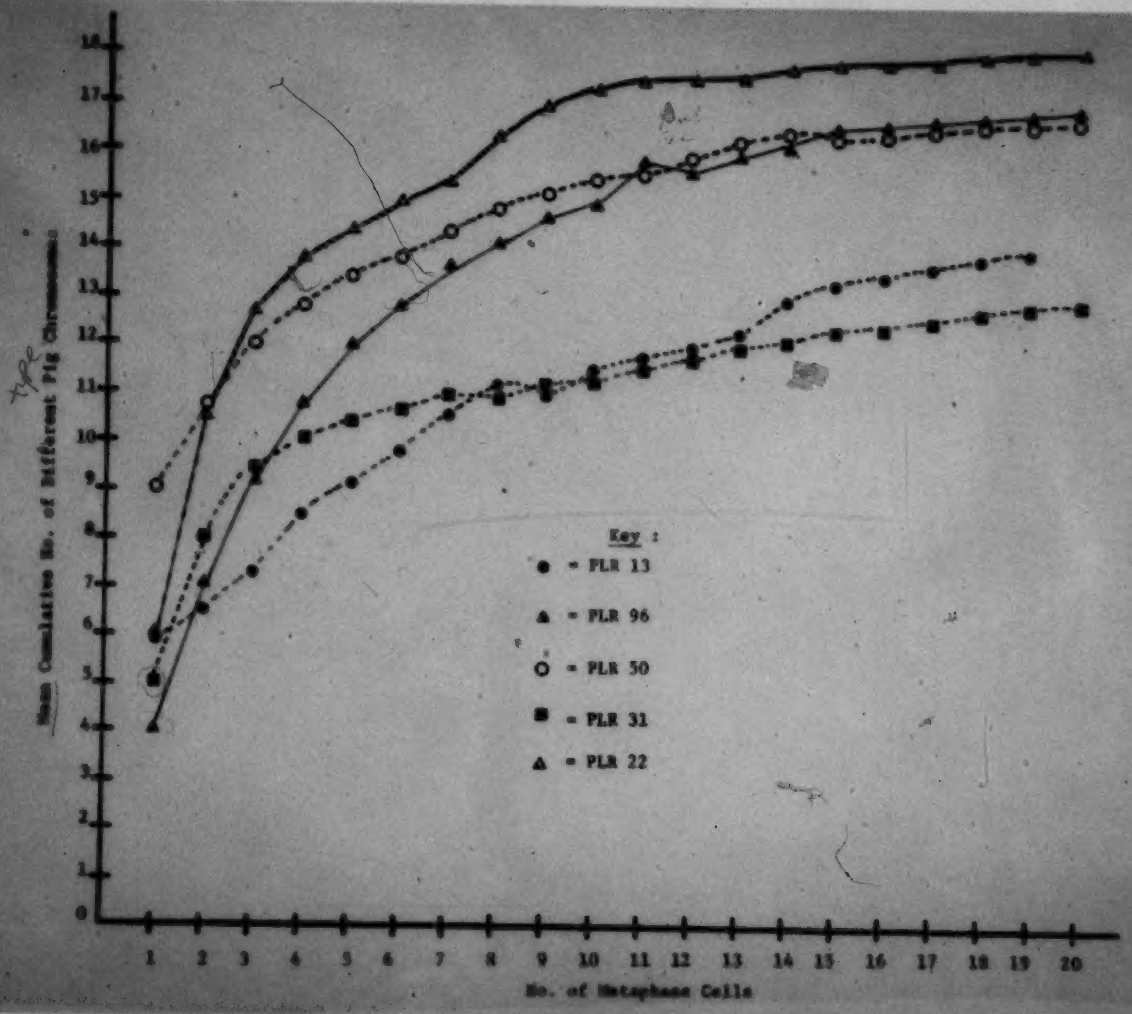

Mean Cumulative No. of Different Pig Chromosomes
type
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
No. of Metaphase Cells
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Key :
● = PLR 13
▲ = PLR 96
○ = PLR 50
■ = PLR 31
△ = PLR 22

Figure 15. Two different parental pig lymphocytes stained with Giemsa and impregnated with silver to reveal the NOR sites.
(a) and (b): Giemsa.
(c) and (d): NORs.
The circles identify No. 10 chromosomes.

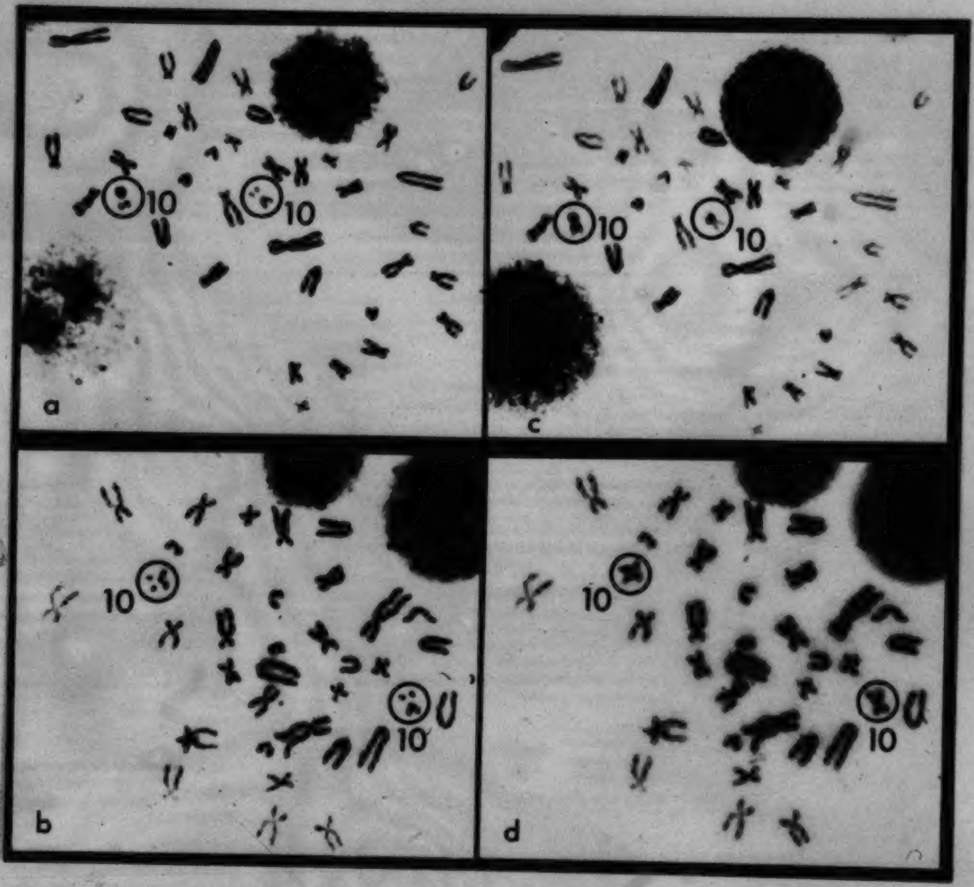

10
10
a
10
10
c
10
10
b
10
10
d

Figure 16. A pig-mouse hybrid cell impregnated with silver to reveal NOR sites. NOR sites in RAG chromosomes: 11 white triangles. Non-detection of NOR sites in pig chromosomes: two No. 10 chromosomes, two white arrows.

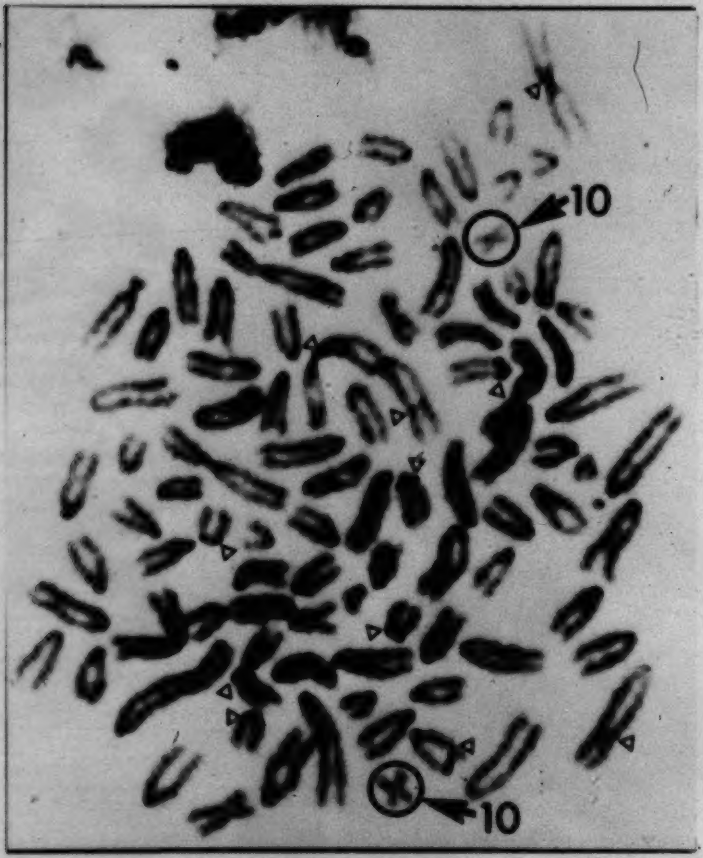
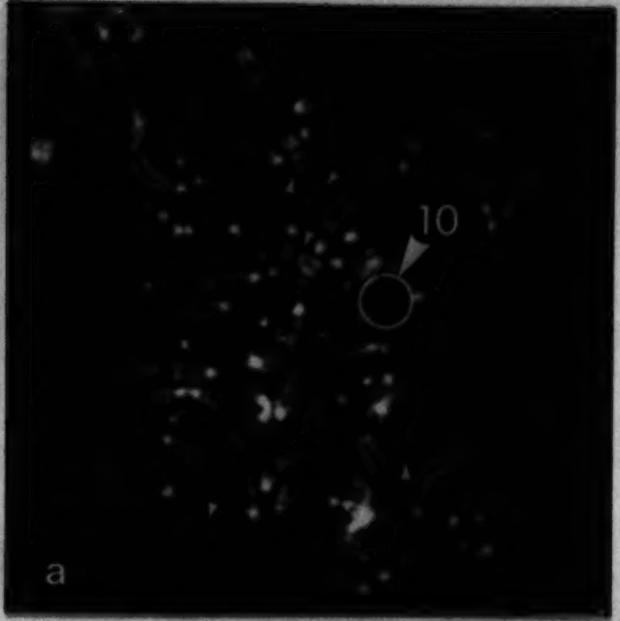

10
10

Figure 17. A pig-mouse hybrid cell stained with actinomycin-D and Hoechst 33258 and impregnated with silver. (a) Fluorescence of mouse centromeres. Non-fluorescence of six pig centromeres: five white triangles and one white arrow to pig chromosome 10. (b) Detection of six mouse NORs: six white triangles. Non-detection of one pig NOR: five black triangles and one black arrow to pig chromosome 10. The four dots in pig chromosome 10 do not represent the NOR. The four chromosomes which do not have fluorescent centomeres (a) and are not marked (b) as pig chromosomes are not identified.


10
a

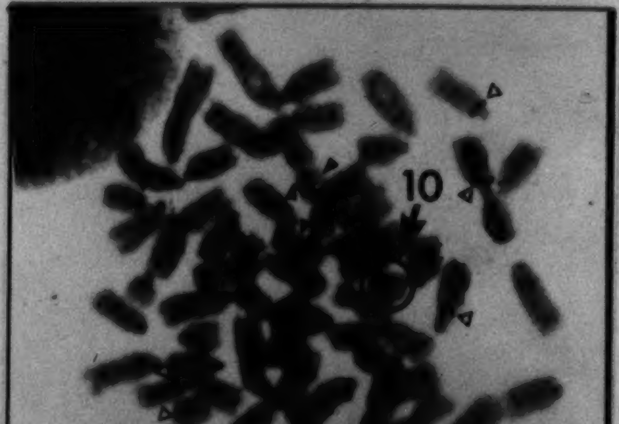

10
b

Figure 18. Pig-mouse hybrid cells impregnated with silver after TPA treatment. (a) After treatment with 100 nM TPA: 12 mouse NORs, white triangles. Non-detection of pig NOR: one No. 10 chromosome, black arrow. (b) After treatment with 200 nM TPA: nine mouse NORs, white triangles. Non-detection of pig NORs: two No. 10 chromosomes, black arrows. One No. 11 chromosome, black arrow, showing that these chromsomes can be discriminated.

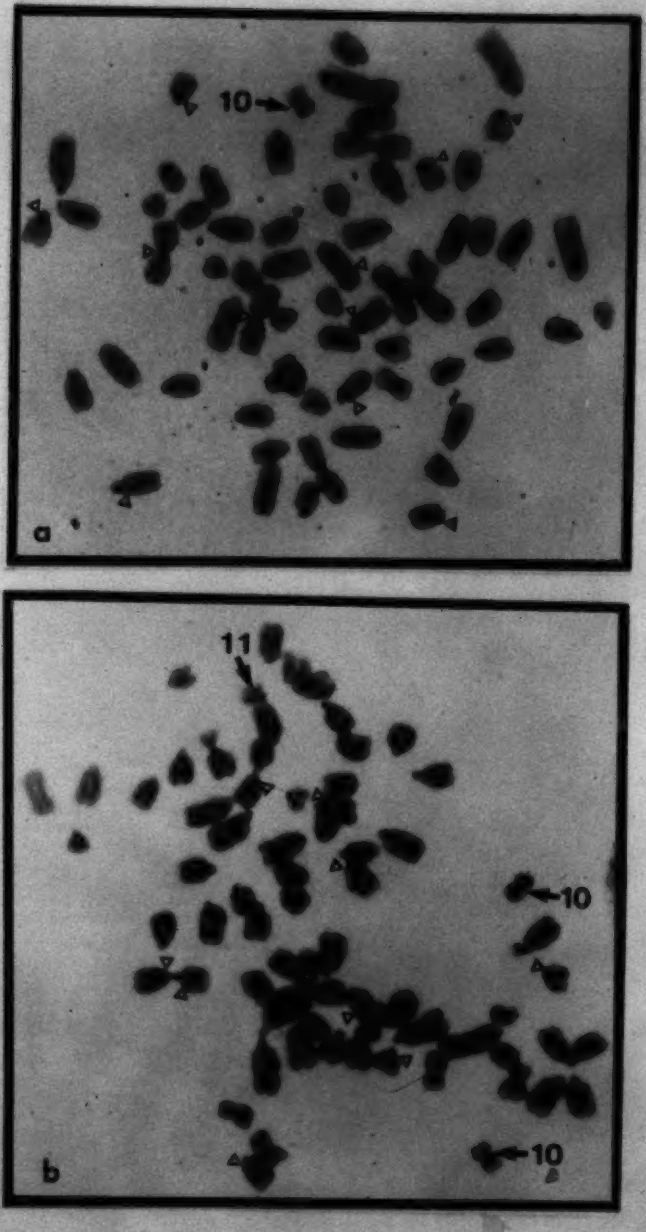
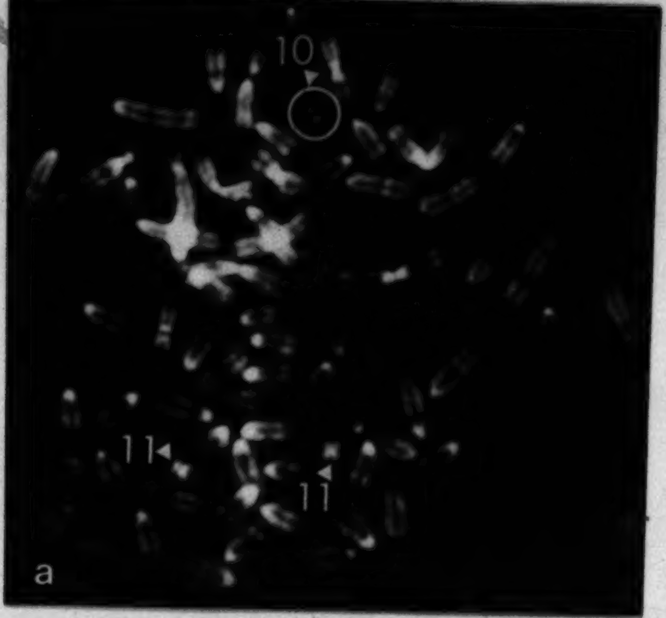

10
a
11
10
10
b

Figure 19. A TPA-treated (100 nM) pig-mouse hybrid cell stained with actinomycin-D and Hoechst 33258 and impregnated with silver.

(a) Fluorescence of mouse centromeres. Non-fluorescence of pig centromeres: two No. 11 and one No. 10 chromosomes, white triangles, showing that these can be discriminated.

(b) Detection of mouse NORs: five are marked, white triangles. Non-detection of pig NOR: two No. 11 and one No. 10 chromosomes, black triangles, showing that these can be discriminated.


10
11
11
a

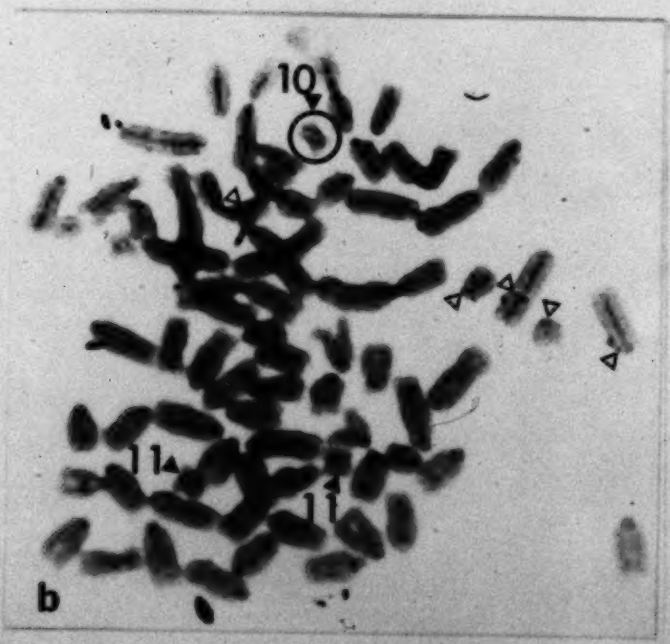

10
11
11
b

Figure 20. Electrophoretic patterns of glucose-6-phosphate dehydrogenase in starch gel.
Channels 1-2: cell lysates from parental RAG cells; mouse G-6PD (RAG).
Channels 3-4: a mixture of parental lymphocyte and RAG cell lysates; mouse and pig G-6PD.
Channels 5-6: cell lysates from a pig-mouse hybrid clone; mouse, intermediate (Het.), and pig ("weak") G-6PD.
Channels 7-8: cell lysates from parental pig lymphocytes; pig G-6PD.

1

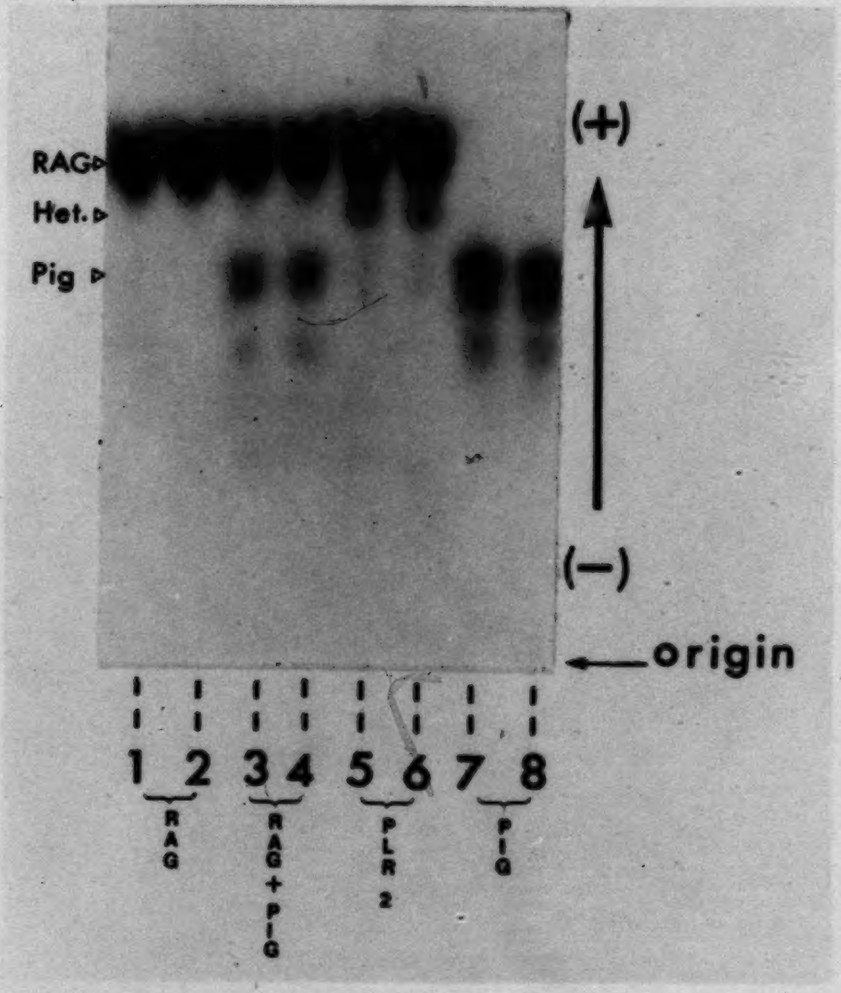

RAG
Het.
Pig
(+)
(−)
origin
1
2
3
4
5
6
7
8
RAG
RAG + PIG
PLR 2
PIG

Figure 21. Electrophoretic patterns of hypoxanthine-guanine phosphoribosyltransferase in starch gel.
Channels 1-2: cell lysates from parental RAG cells; no mouse HPRT.
Channels 3-4: a mixture of parental lymphocyte and RAG cell lysates; pig HPRT.
Channels 5-6: cell lysates from a pig-mouse hybrid clone; pig HPRT.
Channels 7-8: cell lysates from parental pig lymphocytes; pig HPRT.

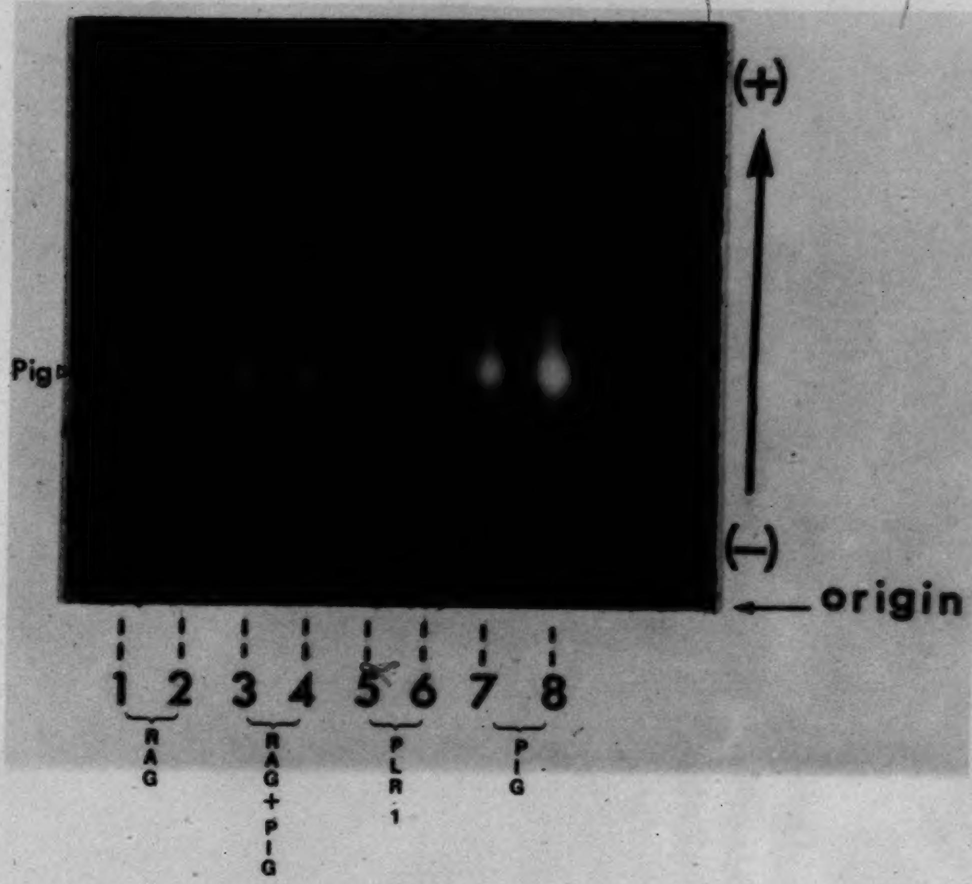

Pig
(+)
(–)
origin
1
2
3
4
5
6
7
8
RAG
RAG+PIG
PLR 1
PIG

Figure 22. Electrophoretic patterns of alpha-galactosidase in starch gel.
Channels 1-4: cell lysates from pig-mouse hybrid clones; mouse GLA (RAG).
Channels 5-8: cell lysates from pig-mouse hybrid clones; mouse, intermediate (Het.), and pig GLA.
Channels 9-10: cell lysates from parental pig lymphocytes; pig GLA ("weak").
Channels 11-12: a mixture of pig liver and RAG cell lysates; pig and mouse GLA.
Channels 13-14: cell lysates from pig liver; pig GLA.
Channels 15-16: cell lysates from parental RAG cells; mouse GLA.

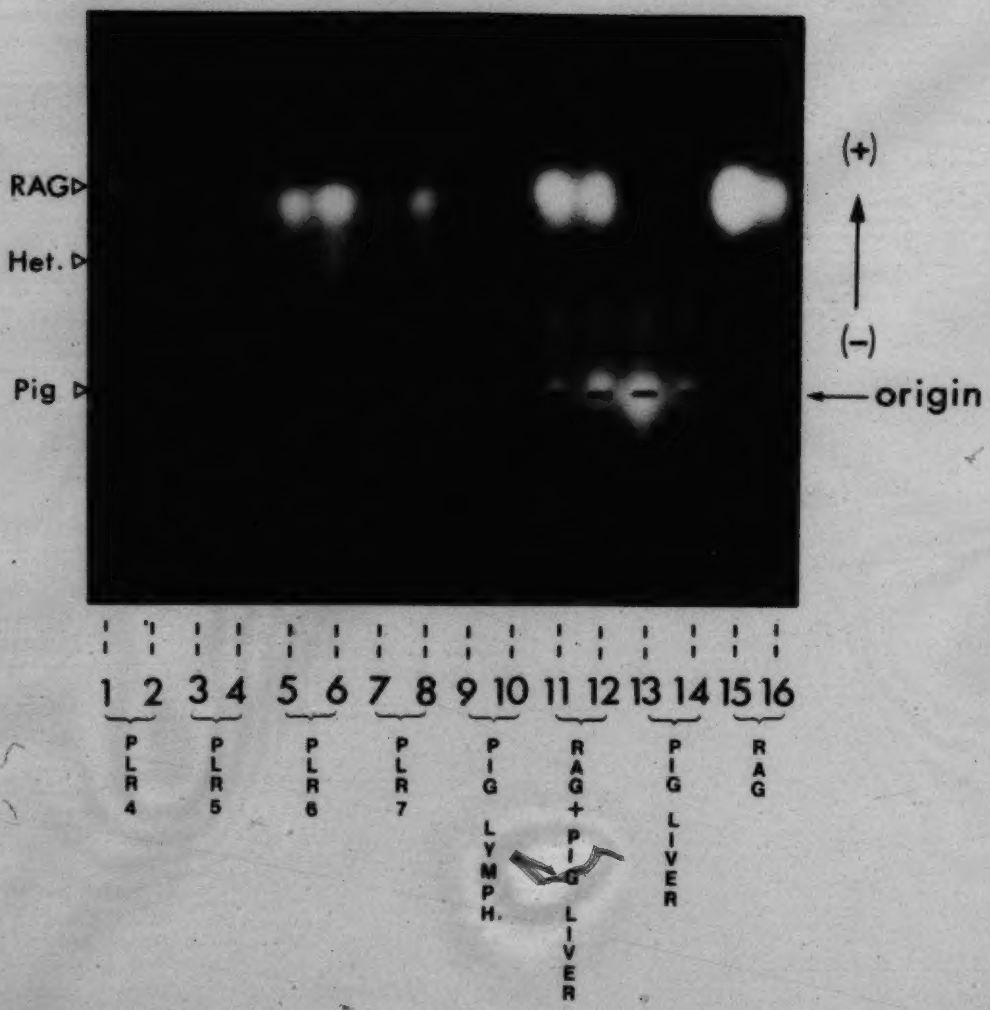

RAG▷
Het. ▷
Pig ▷
(+)
(−)
origin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PLR 4
PLR 5
PLR 6
PLR 7
PIG LYMPH.
RAG + PIG LIVER
PIG LIVER
RAG

Figure 23. Electrophoretic patterns of alpha-galactosidase in starch gel.
Channels 1-7: cell lysates from pig-mouse hybrid clones; mouse GLA (RAG).
Channels 8-9: cell lysates from pig-mouse hybrid clones; mouse, intermediate (Het.), and pig GLA.
Channels 10-11: cell lysates from pig-mouse hybrid clones; mouse GLA.
Channels 12-13: cell lysates from parental pig lymphocytes; pig GLA.
Channels 14-15: cell lysates from parental RAG cells; mouse GLA.

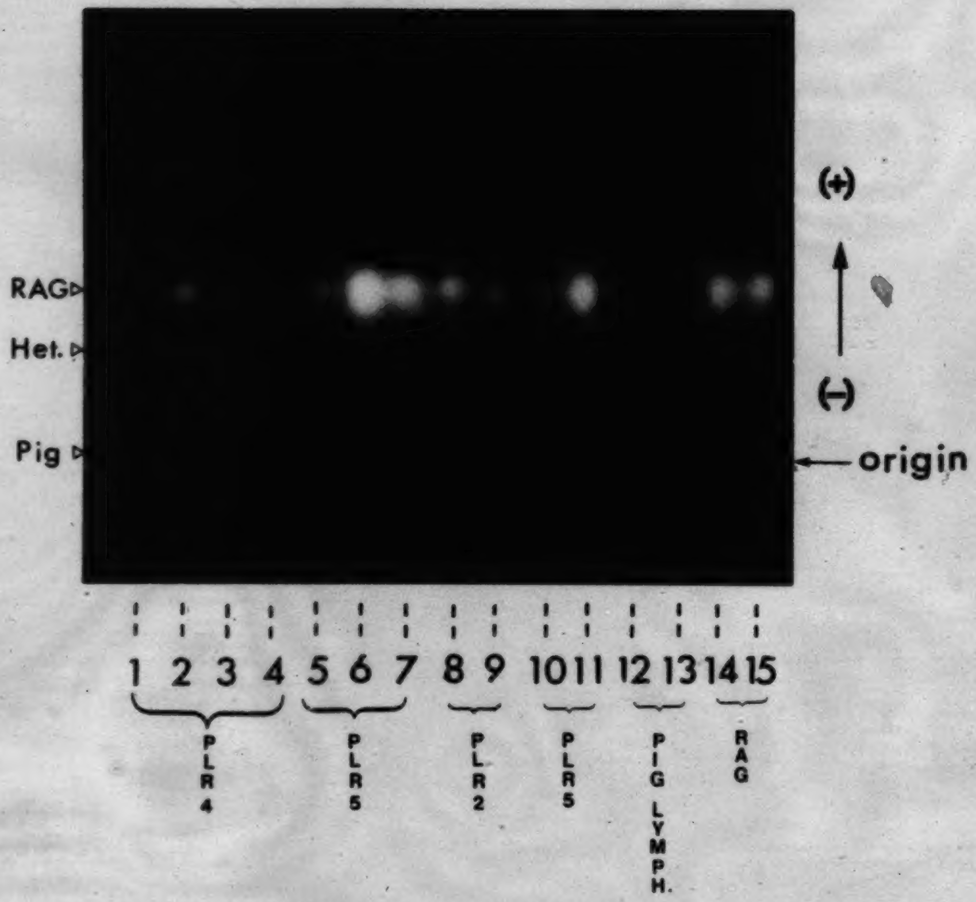
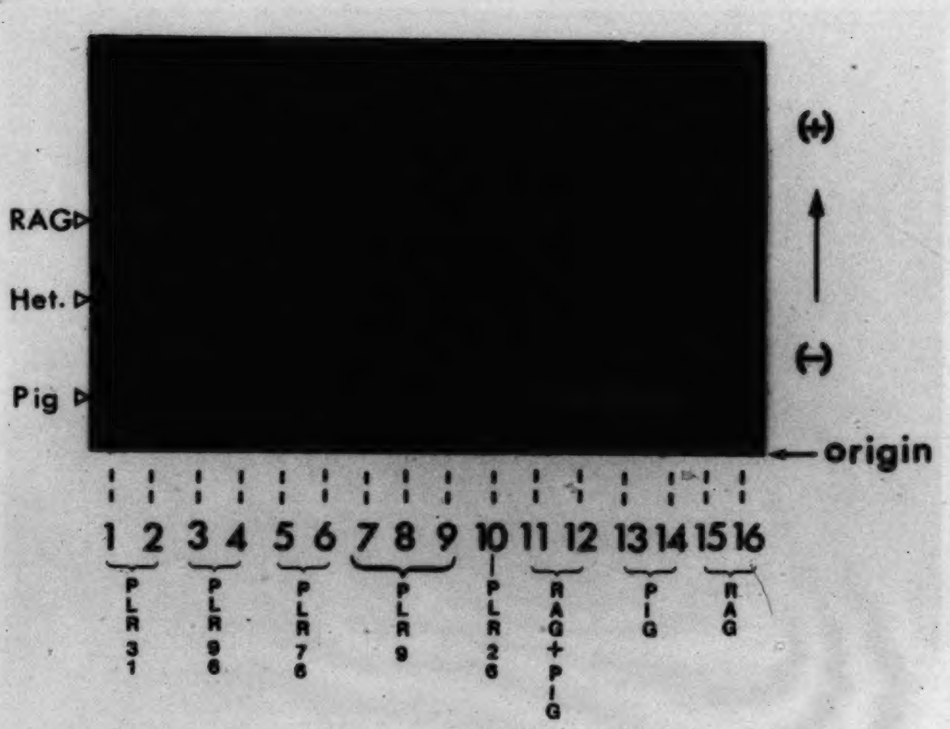
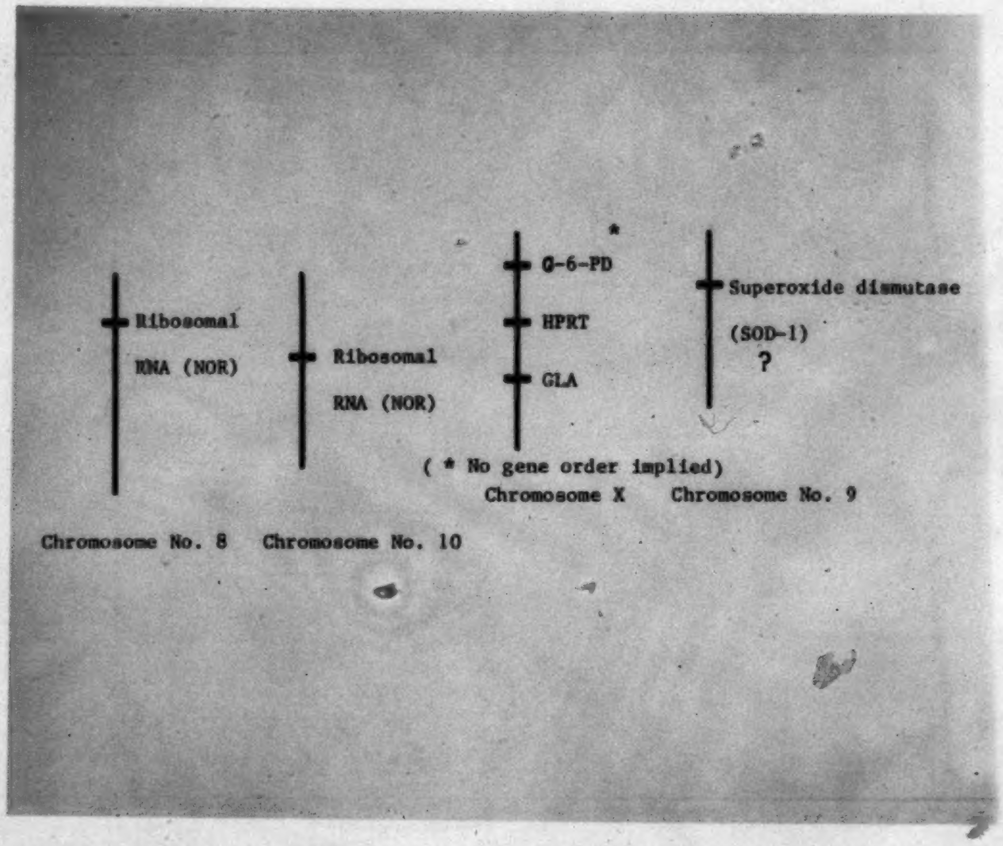

RAG
Het.
Pig
(+)
(–)
origin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
PLR4
PLR5
PLR2
PLR5
PIG LYMPH.
RAG

Figure 24. Electrophoretic patterns of superoxide dismutase.
Channels 1-2: cell lysates from pig-mouse hybrid clones; mouse SOD (RAG).
Channels 3-4: cell lysates from pig-mouse hybrid clones; mouse, intermediate (Het.), and pig SOD.
Channels 5-6: cell lysates from pig-mouse clones; mouse SOD.
Channels 7-9: cell lysates from pig-mouse hybrid clones; mouse, intermediate (Het.), and pig SOD.
Channel 10: cell lysate from a pig-mouse hybrid clone; mouse SOD.
Channels 11-12: a mixture of parental pig lymphocyte and RAG cell lysates; mouse and pig SOD.
Channels 13-14: cell lysates from parental lymphocytes; pig SOD.
Channels 15-16: cell lysates from parental RAG cells; mouse SOD.



RAG
Het.
Pig
(+)
(−)
origin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PLR 31
PLR 96
PLR 76
PLR 9
PLR 26
RAG + PIG
PIG
RAG

Figure 25. Gene assignments to pig chromosomes. These assignments were made during the present study.


Ribosomal
RNA (NOR)
Ribosomal
RNA (NOR)
*
G-6-PD
HPRT
GLA
Superoxide dismutase
(SOD-1)
?
(* No gene order implied)
Chromosome X
Chromosome No. 9
Chromosome No. 8
Chromosome No. 10

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